Evaluation of the therapeutic potential of low dose chronic cannabidiol (CBD) treatment to reverse behavioural deficits of *APP$_{Swe}$/PS1ΔE9* transgenic female mice

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Statement of Authentication

This thesis is submitted in fulfillment of the requirements for the degree of *Master of Research* at Western Sydney University. I, Madilyn Coles, hereby declare that the work submitted in this thesis, entitled '*Evaluation of the therapeutic potential of low dose chronic cannabidiol (CBD) treatment to reverse behavioural deficits of* APP$_{Swe}$/PS1ΔE9 *transgenic female mice'* is originally and authentically the result of my own research endeavour, under the guidance of supervisor Professor Tim Karl. To the best of my knowledge and belief, this thesis contains no materials previously written or published by another person, except where acknowledged in the text of this thesis. I confirm that I have not submitted any material of this thesis for any other degree at this or any other institution.

Signed 

Madilyn Coles

Date 27th November 2019

Dedication

I would like to dedicate this thesis to my late mother, who's support and encouragement throughout my schooling years nurtured my academic abilities and potential, leading me to find my natural drive to follow the path of research, which is where I find myself today. My mother's belief in medicinal cannabis as her choice of therapeutic inspired a passionate interest in the research field of medicinal cannabis, from where the desire to partake in the research topic of this thesis was born.

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Abstract

Introduction: Alzheimer's disease (AD) is a neurodegenerative disease that causes behavioural and cognitive impairments and is hallmarked by amyloid plaques and neurofibrillary tangles of the protein tau. There is currently no cure for AD, with currently available treatments only targeting symptoms. Murine models of AD, such as the *APP$_{Swe}$/PS1ΔE9* (*APPxPS1*) model, replicate important aspects of the disease and allow preclinical insights into the efficacy of other potential therapeutics. With evidence that targeting the endocannabinoid system may be therapeutic in AD, cannabidiol (CBD) has been of recent interest as to whether its anti-inflammatory, antioxidant and neuroprotective properties make it a potential therapeutic avenue for AD. *In vitro* and *in vivo* evidence shows that CBD indeed prevents and reverses AD-related amyloid plaques and neurofibrillary tangles, and cognitive decline including social and object recognition deficits. This thesis aimed to evaluate the therapeutic potential of chronic treatment with low dose CBD (5 mg/kg bodyweight) to reverse the behavioural deficits of *APPxPS1* transgenic mice.

Materials and Methods: 12-month-old control and transgenic female *APPxPS1* mice were treated daily (post-onset of AD-like symptoms) via intraperitoneal injection with 5 mg/kg bodyweight CBD (or vehicle) starting three weeks prior to the assessment of a variety of behavioural paradigms. Mice were tested for anxiety, exploration and locomotion in the light dark test, motor functions including coordination and balance in the pole test and accelerod paradigms, object recognition memory, spatial learning and memory in the cheeseboard test, and sensorimotor gating using the acoustic prepulse inhibition paradigm.

Results: APPxPS1 mice exhibited a hyper-locomotive phenotype in the light dark test, and CBD instigated more explorative-like behaviour in the dark zone in both

genotypes. All mice showed similar motor function, and similar spatial learning rates, although *APPxPS1* mice took longer to complete the cheeseboard training (due to a lower locomotion speed). All mice had intact spatial memory and retrieval memory, although *APPxPS1* mice showed reduced levels of perseverance in the cheeseboard probe trial. Importantly, vehicle-treated *APPxPS1* mice were characterised by object recognition deficits, which CBD recovered without impacting on control mice. Finally, all *APPxPS1* mice exhibited a prepulse inhibition deficit regardless of treatment condition.

Summary and Conclusion: APPxPS1 transgenic mice were hyper-locomotive and CBD elevated exploration in the dark zone of the light dark test. *APPxPS1* females did not exhibit motor function deficits, and importantly CDB did not alter motor function in either *APPxPS1* or control mice. Spatial learning and memory were not affected in *APPxPS1* transgenic females. Importantly, 5 mg/kg CBD reversed novel object recognition deficits in *APPxPS1* transgenic females suggesting a therapeutic-like effect in this established mouse model for AD. CBD did not reverse PPI deficits evident in transgenic females. Further research into the effects of CBD should consider investigating molecular mechanisms as well as testing other treatment designs including the consideration of other doses and ages of test animals. In conclusion, this study suggests that CBD has therapeutic value for particular behavioural impairments present in AD patients.

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List of abbreviations

2-AG	2-arachidonylglycerol
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AD-DS	Alzheimer's disease in Down syndrome
AICD	Amyloid precursor protein intracellular domain
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APPxPS1	*APP_{Swe}/PS1ΔE9*
ASR	Acoustic startle response
Aβ	Amyloid-beta
$Aβ_{40}$	Amyloid-beta peptide of 40 residues
$Aβ_{42}$	Amyloid-beta peptide of 42 residues
CB	Cheeseboard
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBD	Cannabidiol
CNS	Central nervous system
αCTF	Alpha C-terminal fragment
βCTF	Beta C-terminal fragment
ECS	Endocannabinoid system
ELISA	Enzyme-linked immunosorbent assay

EPM	Elevated plus maze
FAAH	Fatty acid amide hydrolase
FAD	Familial Alzheimer's disease
FTD	Frontotemporal dementia
IBA-1	Ionized calcium binding adaptor molecule-1
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
i.p.	Intraperitoneal
ISI	Inter-stimulus interval
ITI	Inter-trial interval
LD	Light dark test
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
MWM	Morris water maze
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NORT	Novel object recognition task
OF	Open field
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PPARγ	Peroxisome proliferator-activated receptor-gamma
PPI	Prepulse inhibition

PS1	Presenilin 1
PS2	Presenilin 2
rCB	Reversal cheeseboard
RM	Repeated measures
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAD	Sporadic Alzheimer's disease
sAPPα	Soluble amyloid precursor protein alpha
sAPPβ	Soluble amyloid precursor protein beta
SEM	Standard error of means
THC	Δ_9-tetrahydrocannabinol
TNF-α	Tumour necrosis factor alpha
VEH	Vehicle
WT	Wild type-like

Chapter 1: Introduction

1.1 Alzheimer's disease

As a result of Australia's ageing population, it is expected that by 2056 around 6.4 million Australians will have been diagnosed with dementia, which is currently incurable and without effective preventative options (Brown, Hansnata & La 2017). Although dementia is not considered part of normal ageing, it primarily affects the elderly (AIHW 2012), with age being the most important risk factor (Chen & Mobley 2019). As an umbrella term, dementia describes a group of neurodegenerative diseases characterised by the irreversible and progressive decline in cognitive skills, including impairments in language, memory, perception and personality. Accounting for up to 75% of all cases worldwide, Alzheimer's disease (AD) is the most common form of dementia, and, following coronary heart disease, dementia including AD was the second leading cause of death in Australia in 2017, especially among the elderly (AIHW 2019). It is predicted that without significant advancement in diagnostic and therapeutic technologies, the economic cost of the disease to the Australian health and aged care systems will exceed $1 trillion over the next four decades (Brown, Hansnata & La 2017).

AD is an insidious neurodegenerative disease that is caused by progressive damage to neuronal cells and results in varying concomitant symptoms related to the area of affected brain. Mild cognitive impairment (MCI) generally precedes AD, with an average of 32 % of individuals with MCI developing AD within 5 years (Alzheimer's Association 2018). AD progresses from mild, to moderate and finally to severe AD. In the mild stage, AD is initially characterised by apathy, depression and mild deficits in short-term memory, spatial orientation, learning and communication. Occasionally, it can be difficult to distinguish these early signs of AD from typical age-related

cognitive changes (Alzheimer's Association 2018). As AD progresses to moderate clinical stages, memory continues to decline and begins to affect everyday tasks including reading, writing, eating, dressing and bathing, and results in agitation, suspiciousness and loss of emotional control. Patients living with the late stages of the disease require full-time care, and have severe disturbances to cognitive abilities including disorientation, poor judgement, behavioural changes, severe impairments in speech and facial recognition, and susceptibility to secondary diseases. Ultimately, AD results in loss of bodily functions, difficulty speaking, swallowing and walking, and is fatal, commonly due to aspiration pneumonia, which is the result of a difficulty in swallowing combined with being bed-bound (Alzheimer's Association 2018).

Diagnosis of AD is often difficult and requires a variety of diagnostic approaches including blood tests, physical and neurological examinations, brain imaging to determine the levels of AD-relevant markers, and consideration of medical and family history of patients. However, often a verified diagnosis can only be made post-mortem, and involves analysis of brain tissue, as the brain is the main organ affected in AD. A pathological hallmark of AD is the extracellular accumulation of amyloid-beta (Aβ) protein fragments around the neurons in the brain, which are the result of the aberrant cleavage of amyloid precursor protein (APP). These fragments form Aβ plaques that interfere with neuronal communication, which is thought to cause cell death and the signs and symptoms of AD (Alzheimer's Association 2018). However, while amyloid burden is a cardinal feature of AD, there is no correlation between the amount of amyloid burden, and the severity or stage of AD (Chen & Mobley 2019). Another hallmark of AD is the intracellular accumulation of hyperphosphorylated microtubule associated protein tau (MAPT), forming neurofibrillary tangles (NFT) that block the transport of nutrients within neurons. Cerebral atrophy is also a very characteristic sign

of AD in post-mortem brains and is caused by neuronal cell death. Furthermore, microglial activation, oxidative stress and chronic inflammation of the brain are seen in AD patients due to the activation of the immune system in response to toxic Aβ plaques and NFTs (Alzheimer's Association 2018). Damage to the brain of someone affected by AD on average starts 15 years before symptoms, which manifest once the brain is no longer able to compensate with the early damage. The neuropathology of AD and the roles of amyloid plaques and NFTs are further described in section 1.1.2.

AD seems to affect women disproportionately. Briefly, AD is significantly more common in women than men (Oveisgharan et al. 2018) and studies have found that women have poorer cognitive profiles than in men at the same stage of AD (Laws, Irvine & Gale 2016). Also, a recent study has found that women with AD have more NFTs and slightly more Aβ compared to men (Oveisgharan et al. 2018), demonstrating the presence of sex differences in AD pathologies. Briefly, it has been suggested that the sex differences seen in AD may be in part due to a reduction of estrogen in postmenopausal women, the greater cognitive reserve in men, and the influence of the apolipoprotein E *e4* allele (a risk factor for AD, introduced below in section 1.1.1; Laws, Irvine and Gale (2016)).

1.1.1 Genetics of AD

Presently, there are numerous categorisations of AD. Most often, AD is categorised as either early-onset familial AD (FAD) or late-onset sporadic AD (SAD), depending on the age of onset and the underlying cause and genetics. However, while the causes of FAD and SAD are dissimilar, the two are clinically and histopathologically indiscernible (Götz & Ittner 2008). Of SAD and FAD, late-onset SAD is the more common form of AD, with disease onset occurring at the age of 65 years or older. The cause of SAD is still unclear, although risk factors for SAD

including susceptibility genes and environmental factors have been identified. Some metabolic risk factors for SAD include hypertension, obesity and type 2 diabetes mellitus (Mendiola-Precoma et al. 2016). The most widely studied genetic risk factor for SAD is the gene encoding apolipoprotein E (*APOE*), which has been identified as a susceptibility gene in genome-wide association studies, as reviewed (Mendiola-Precoma et al. 2016). APOE transports cholesterol in the blood and has three forms, the *e2*, *e3* and the *e4* form. All individuals inherit two copies of *APOE*. The *e4* variant of the gene (*APOE-e4*) is the form associated with an increased risk of AD and is linked to developing AD at an earlier age of onset compared to individuals possessing the *e2* and *e3* variants. Possessing one copy of *APOE-e4* results in a three times higher chance of developing AD compared to someone with two copies of the *e3* variant (the most common variant), while possessing two copies of *APOE-e4* increases this risk to 8 to 12 times. Those possessing the *e2* variant may have a decreased risk of developing AD compared to those possessing the *e3* form (Alzheimer's Association 2018). It is important to note that inheritance of the *APOE-e4* gene does not cause AD, it merely increases ones risk of developing the disease (Alzheimer's Association 2018; Mendiola-Precoma et al. 2016).

Early-onset FAD is less common than SAD and is estimated to represent less than 1% of all AD cases. FAD results from the inheritance of an autosomal dominant mutation in the genes encoding APP, presenilin 1 (PS1) or presenilin 2 (PS2), the latter two being enzymes participating in the processing of APP. Inheritance of AD-relevant mutations in *APP* or *PS1* guarantees the disease, while inheritance of a mutation in *PS2* results in a 95% chance of developing AD (Alzheimer's Association 2018). To date, 52 *APP* mutations (26 duplication mutations and 26 missense mutations; Julia and Goate (2017)), over 180 *PS1* mutations and around 10 *PS2* mutations have been

linked to AD. Individuals that inherit any of these three AD-relevant mutations often develop symptoms of AD before the age of 65, usually in their 40's or 50's, but sometimes as early as 30 years of age, hence the term early-onset FAD. Mutations in *APP*, *PS1* and *PS2* result in the aberrant cleavage of APP into Aβ peptides of 40 residues ($A\beta_{40}$) or of 42 residues ($A\beta_{42}$), which are thought to form toxic Aβ plaques responsible for causing neuronal cell death (Alzheimer's Association 2018). This is further discussed in section 1.1.2.

Other than SAD and FAD, AD can also be categorised in other ways. Young-onset AD, which is distinct from early-onset FAD, results in disease development before the age of 65 without any known genetic risk factor or family history present (Chen & Mobley 2019), i.e. young-onset AD is sporadic while FAD is the result of the inheritance of a mutation, however both result in disease development before the age of 65 years. In other words, young-onset AD is similar to late-onset SAD in that it is sporadic, however it is seen in people of a much younger age than those with SAD. Furthermore, AD can also be seen in Down syndrome (known as AD-DS), where 50% of individuals with Down syndrome develop AD within their lifetime. The extra copy of chromosome 21, which contains the gene for APP, results in the production of more wild type levels of the protein and Aβ fragments in Down syndrome individuals, leading to AD-DS (Alzheimer's Association 2018; Chen & Mobley 2019).

1.1.2 Neuropathology of AD

There are numerous hypotheses as to the pathological course of AD, and the role of mutations in *APP*, *PS1* and *PS2* in AD. The *amyloid cascade hypothesis*, first proposed by Hardy and Higgins (1992), has been the most prominent hypothesis in explaining the cause of AD, and suggests that the deposition of Aβ is the initial pathological event in AD, which then leads to amyloid plaques, NFT and eventually

dementia (Reitz 2012). It can be summarised as follows (Figure 1): mutations in *APP*, *PS1* or *PS2* result in increased Aβ production, which leads to oligomerisation of $A\beta_{42}$ and plaque formation. This leads to microglial activation and progressive injury to neurons and synapses, which then cause altered homeostasis of neurons and oxidative injury. The activity of phosphatases and kinases becomes altered and leads to NFT, which finally leads to widespread neuronal dysfunction and cell death, resulting in dementia (Julia & Goate 2017). The neuropathological components of AD implicated in the *amyloid cascade hypothesis* are further discussed below.

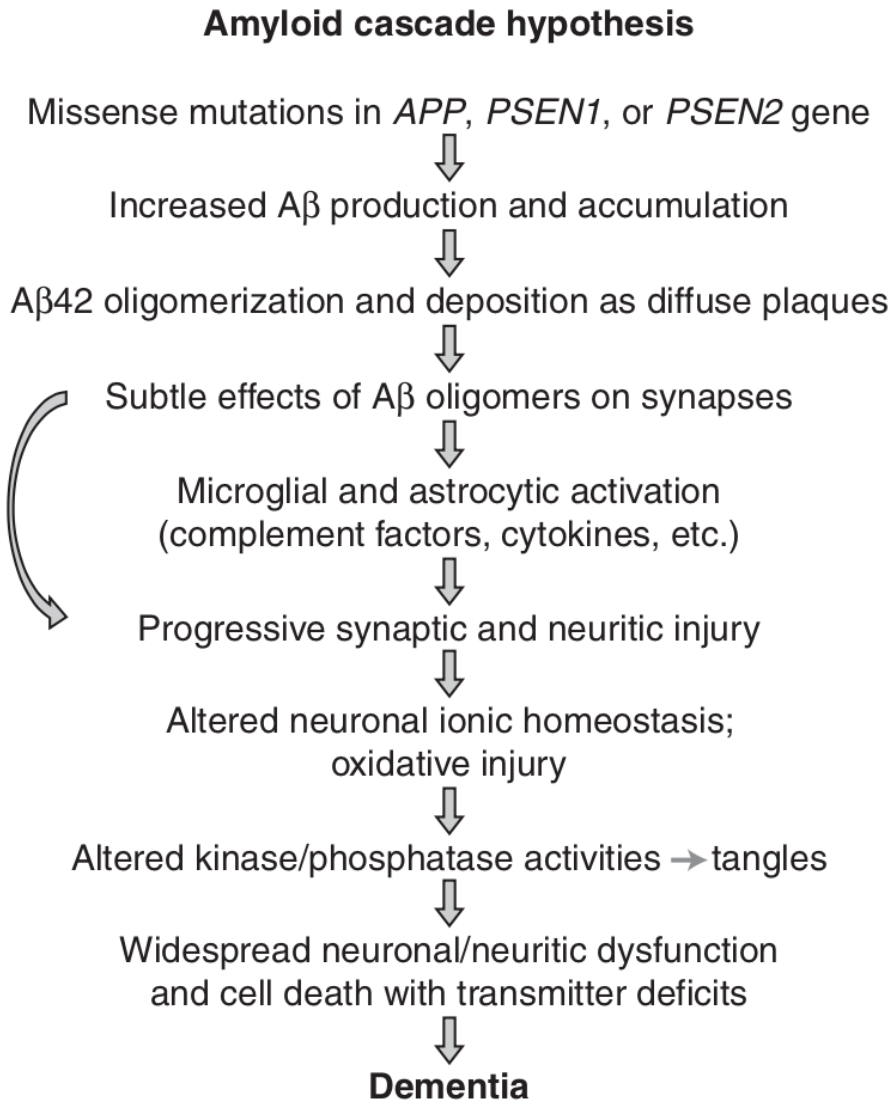

Figure 1: The *amyloid cascade hypothesis*. The proposed sequence of pathogenic events leading to AD. The *amyloid cascade hypothesis* begins with mutations in amyloid precursor protein (*APP*), presenilin 1 (shown as *PSEN1)* or presenilin 2 (shown as *PSEN2*) that cause accumulation of amyloid-beta (Aβ) and initiate a cascade that leads to dementia. *Abbreviation*: Amyloid-beta peptide of 42 residues ($A\beta_{42}$). Image from Julia and Goate (2017).

As mentioned, extracellular plaques consisting of deposited Aβ peptides are a key pathological sign of AD; these plaques are thought to be neurotoxic and interfere with neuronal communication, and are thought to be the result of the aberrant splicing of APP. APP is a transmembrane protein found in cells throughout the body and is encoded by *APP* on chromosome 21, which consists of 18 exons that are alternatively

spliced to produce APP proteins that are between 695 and 770 amino acids in length (Julia & Goate 2017). APP is sequentially cleaved by the enzymes α-, β- and γ-secretases, in two principle pathways: the non-amyloidogenic pathway (Figure 2A) and the amyloidogenic pathway (Figure 2B). The majority of APP processing in the healthy brain is via the non-amyloidogenic pathway, which involves the cleavage of APP by α-secretase. This forms an extracellular N-terminal fragment known as soluble APP alpha (sAPPα), and a cell membrane bound C-terminal fragment known as αCTF. αCTF is then cleaved by γ-secretase, producing the APP intracellular domain (AICD), which regulates gene transcription, and the extracellular P3 peptide. *PS1* and *PS2* encode the catalytic components of γ-secretase. The alternate and less common pathway, the amyloidogenic pathway, is involved in the generation of amyloid peptides. This occurs by cleavage of APP by β-secretase, forming the extracellular soluble APP alpha fragment (sAPPβ) and the C-terminal fragment beta (βCTF). γ-secretase then cleaves βCTF to produce AICD and extracellular plaque-forming Aβ peptides (Chen & Mobley 2019). The amyloid peptides produced in the amyloidogenic pathway vary in length, as the site of γ-secretase cleavage is promiscuous (Julia & Goate 2017). The majority of amyloid peptides formed in the amyloidogenic pathway are 40 amino acids long ($A\beta_{40}$), with around 10% of peptides being 42 residues long ($A\beta_{42}$). $A\beta_{42}$ peptides more readily aggregate into fibrils and plaques (Julia & Goate 2017) due to the C-terminal isoleucine and alanine residues that give this peptide hydrophobic properties. For this reason $A\beta_{42}$ is predominately found in cerebral plaques in AD (Chen & Mobley 2019).

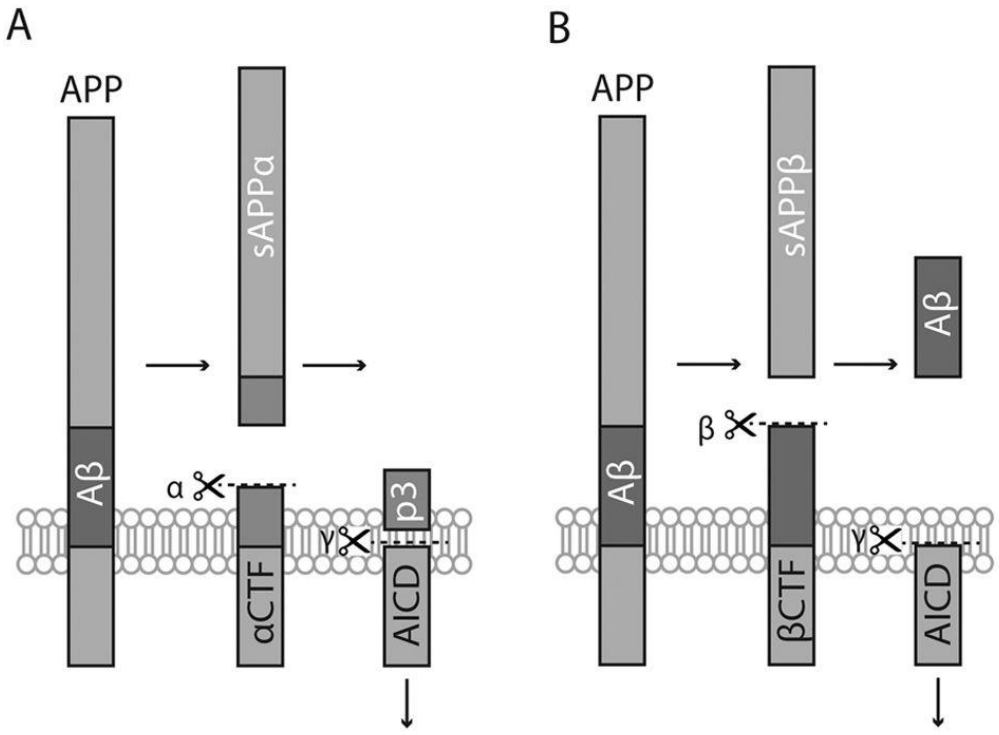

Figure 2A-B: Amyloid precursor protein (APP) processing.
A) The non-amyloidogenic pathway is the major processing pathway of amyloid precursor protein (APP) and **B)** the amyloidogenic pathway that results in the production of amyloid beta (Aβ) peptides. Scissor icon represents a secretase enzyme. *Abbreviations:* Amyloid precursor protein intracellular domain (AICD), alpha C-terminal fragment (αCTF), beta C-terminal fragment (βCTF), P3 peptide (P3), soluble amyloid precursor protein alpha (sAPPα), soluble amyloid precursor protein beta (sAPPβ). Diagram adapted from Bergström et al. (2016).

Mutations in *APP*, *PS1* and *PS2* seem to influence the overproduction of the more toxic $A\beta_{42}$. For example, pathogenic missense mutations close to the cleavage sites of β- or γ-secretases within or immediately flanking the Aβ domain of *APP* (encoded by exons 16 and 17) can result in either i) overall elevated production of Aβ peptides, ii) mutated Aβ peptides that form fibrils faster than wild type peptides, or iii) impaired secretase cleavage and increased hydrophobicity of the produced Aβ peptides, all of which are associated with aggregation of the peptides and the formation of AD-characteristic plaques. Missense mutations can also cause increased deposition of $A\beta_{40}$ in vascular walls, which is associated with significant cerebral amyloid angiopathy and

haemorrhagic stroke (Julia & Goate 2017). Further, a double mutation in exon 16 of *APP* in a Swedish family of FAD appears to result in a higher production of both $A\beta_{40}$ and $A\beta_{42}$ (Julia & Goate 2017). Other mutations in *APP* seem to be protective of AD and confirm the role of amyloid deposition in the pathogenesis of AD, which is currently debated. For example, an alanine-to-threonine substitution adjacent to the β-secretase cleavage site in APP results in approximately a 40% reduction in the formation of Aβ peptides *in vitro* as this mutation prevents cleavage of APP by β-secretase, thereby shunting APP processing into the non-amyloidogenic pathway, reducing Aβ aggregation, and thus reducing the risk of AD (Julia & Goate 2017).

How mutations in *PS1* and *PS2*, encoding the catalytic core of γ-secretase, contribute to the cause of AD is still poorly understood, with both toxic gain-of-function and loss-of-function hypotheses being investigated. Regardless of the function of the mutation, *in vivo* and *in vitro* paradigms have shown that FAD-relevant *PS1* and *PS2* mutations seem to increase the relative amount of the toxic $A\beta_{42}$ versus $A\beta_{40}$ in many but not all cases, which is more likely due to decreasing the total amount of $A\beta_{40}$ rather than increasing the total amount of $A\beta_{42}$ (Chávez-Gutiérrez et al. 2012; Kelleher & Shen 2017).

While the *amyloid cascade hypothesis* implicates amyloid plaques as the neurotoxic initiator of AD, there is debate as to whether these plaques are indeed damaging, or whether they are actually a neuroprotective adaptation (Zhou et al. 2018). It has been argued that the historical data that scientists have used to conclude that Aβ plaques are neurotoxic, could also support the converse idea that disease in AD, for example cellular stress, leads to increased Aβ as a protective function; i.e. amyloid plaques may be a response to AD, not the cause (Lee et al. 2004). Evidence for this is the fact that cellular stress decreases in response to increased Aβ, and that Aβ can act

as a superoxide dismutase (Lee et al. 2004). Additional evidence for this challenge to the dogma includes the fact that Aβ does not correlate with cognitive decline in AD, that Aβ occurs late in the pathogenesis of AD, that there is no evidence for amyloid toxicity *in vivo* (only *in vitro*), and the suggestion that the increase in Aβ associated with genetic mutations linked to AD could be explained as "*mutation leads to disease leads to increased amyloid-β*" (Lee et al. 2004). As a result, it is now generally agreed that rather than amyloid plaques, it is soluble dimers of Aβ that are neurotoxic (Zhou et al. 2018). From this comes the idea that the accumulation and sequestration of soluble Aβ via the amyloid plaques might be the body's protective mechanism to reduce the toxic forms in the brain (Treusch, Cyr & Lindquist 2009). It is therefore important to note that if amyloid plaques are a protective adaptation in AD, then therapies designed to reduce plaque formation could be detrimental. Nevertheless, regardless of the toxicity or function of amyloid plaques, they are one of a few distinct hallmark features of AD.

Neuroinflammation is also seen in AD. Microglia, the resident macrophage-like immune cells of the central nervous system (CNS), are noticeable in areas of neurodegenerative damage in AD. Microglia are activated in response to receptor ligation to Aβ, and in the healthy brain microglia clear Aβ before plaque formation begins via phagocytosis of Aβ and through secretion of pro-inflammatory cytokines and chemokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). In AD, microglia are unable to completely clear the area of Aβ due to incapable phagocytic abilities, possibly due to the down-regulation of Aβ phagocytosis receptor expression (Heneka et al. 2015). This results in a build-up of Aβ, microglia and inflammatory cytokines in affected brain regions. This prolonged microglial activation and persistent exposure to pro-inflammatory cytokines leads to neuroinflammation

which may also be itself a pathological contributor to AD. This process causes functional and structural damage to neurons, with neuronal debris leading to perpetuation of inflammation, which exacerbates disease progression (Heneka et al. 2015). It is thought that perpetuation of these processes over time may lead to the irreversible senescence of microglia (Heppner, Ransohoff & Becher 2015).

In addition, impairments to glutamatergic and cholinergic signalling are seen in AD, both of which are involved in learning and memory processes, as reviewed (Parsons et al. 2013). The release of cytokines TNF-α and IFN-γ during prolonged microglial activation at Aβ plaque sites in AD create an excito-neurotoxic state, which leads to further neurodegeneration and induces the release of the excitatory neurotransmitter glutamate. This leads to the overstimulation of N-methyl-D-aspartate (NMDA) receptors and to the subsequent sustained influx of calcium ions. This increase in calcium creates a pathological "background noise" that impairs signalling cascades leading to impaired long-term potentiation and thereby impaired learning and memory (Parsons et al. 2013). Increased calcium also leads to elevated production of nitric oxide (NO), which inhibits energy production by mitochondria, thereby leading to impaired neuronal function and neurodegeneration at regions of microgliosis (at Aβ plaque sites). Increased NO, and other reactive oxygen species (ROS), cause oxidative damage to neurons at these sites as the antioxidant systems are overwhelmed and no longer able to mediate the effects of ROS in AD (Takeuchi 2010). This leads to altered oxidative homeostasis and oxidative stress, which is thought to contribute to the downstream hyperphosphorylation of tau. Furthermore, AD causes the degeneration of cholinergic neurons in the basal forebrain, which have projections in areas of the brain important for memory including amygdala, hippocampus and frontal cortex (Schliebs & Arendt 2011). This leads to long-term disruption to acetylcholine (ACh)

neurotransmission and contributes to cognitive impairment, as ACh is an essential CNS neurotransmitter involved in cognition, learning and memory (Parsons et al. 2013). Numerous post-mortem studies demonstrate that the decline in ACh levels that contribute to cognitive decline in AD are linked to the decrease of choline acetyltransferase activity (which is involved in the synthesis of ACh), decreases in ACh release, decreases in nicotinic and muscarinic ACh receptor binding and perhaps alterations to acetylcholinesterase (AChE; the enzyme involved in ACh metabolism) activity, as reviewed (Auld et al. 2002; Lombardo & Maskos 2015; Parsons et al. 2013; Schliebs & Arendt 2011). Disruptions to glutamatergic and cholinergic signaling in AD are important factors in the current treatment strategies for AD, discussed further in section 1.1.3.

Another key pathological sign of AD is the occurrence of NFTs, which are composed primarily of tau (MAPT), encoded by the *MAPT* gene. In a healthy cell, tau is involved in cytoskeletal stabilisation and promotes the formation of microtubules, which is achieved through phosphorylation of 2-3 tau residues. However, in AD, tau becomes hyperphosphorylated and aggregates together, forming NFTs. This resulting pathology is neurotoxic and linked to neurodegeneration, not only due to the formation of insoluble intracellular fibrils, but also due to the reduced affinity of tau to bind to microtubules, resulting in a destabilised cytoskeleton and reduced axonal transport of organelles and biomolecules (Shepherd, McCann & Halliday 2009). Although there are currently no mutations in *MAPT* that are linked to AD, mutations in *MAPT* are indeed associated with the cause of another dementia, frontotemporal dementia (FTD). Like AD, FTD is characterised by NFTs, and transgenic animal models of FTD expressing mutations in *MAPT* demonstrate that NFTs induce cognitive deficits, indicating that NFTs are linked to cognitive decline in dementia (Eersel et al. 2015).

Also, transgenic models of tau pathology develop NFTs in the absence of amyloid pathology, indicating that amyloid pathology in AD may induce NFTs, but NFTs do not induce amyloid pathology, congruent with the *amyloid cascade hypothesis* and the suggestion that tau pathology occurs downstream of amyloid pathology (Kitazawa, Medeiros & M LaFerla 2012). Furthermore, tau pathology is more commonly seen in later stages of AD (Kuret et al. 2005).

1.1.3 Treatment of AD

There is currently no cure for AD, and with only a limited number of therapeutic options currently available that act to treat the symptoms of the disease, there is a dire need for more research into possible therapies to prevent, treat and cure the disease. Currently available treatments include three AChE inhibitors and one NMDA receptor antagonist that only provide symptomatic relief to patients in early disease stages without altering disease progression and also have numerous side effects. Rather than only treating symptoms of AD, treatments that focus on targeting the pathology of AD would be more beneficial in the attempt to treat and cure the disease. Indeed, clinical trials are currently underway to investigate the potential of aetiology-based treatments that aim to block the generation of the toxic amyloid and tau pathologies associated with the progression of AD (Cummings et al. 2019). Furthermore, complementary preventative treatments aiming to manage comorbidities and advise healthy lifestyles including advice on diet, cognitive stimulation and exercise are being investigated (Mendiola-Precoma et al. 2016).

Four approved pharmacological therapies are available and as mentioned only treat the symptoms of the disease without preventing or reversing the disease pathology. Memantine, a non-competitive NMDA receptor antagonist is used to inhibit the glutamatergic system to reduce cell death and has been approved for those with

moderate or severe AD. However, side effects such as dizziness, fatigue, vomiting and high blood pressure have been reported, and the effectiveness of the drug reduces after a few years (Wong 2016). Donepezil, rivastigmine and galantamine are AChE inhibitors, which prevent the loss of ACh from neurons and improve the cognition and daily life of those with mild to moderate AD. Again, these are associated with side effects such as diarrhoea, vomiting, nausea, dizziness, insomnia and fatigue, as reviewed (Wong 2016). Clinical observations indicate that the complementary mechanisms of memantine and AChE inhibitors confers potential for their use in combination, with preclinical studies showing that combination therapy can produce greater improvements in memory than either treatment alone, as reviewed in Parsons et al. (2013).

As mentioned, clinical trials investigating disease-modifying treatments are currently underway and are based on the *amyloid cascade hypothesis*. These initiatives focus on preventing amyloid burden by targeting secretase activity or binding amyloid, but also evaluate the potential of preventing NFT by targeting the kinases involved in the hyperphosphorylation of tau. For example, metalloproteinases that upregulate and stimulate α-secretase and the non-amyloidogenic pathway are being investigated. γ-secretase inhibitors and modulators have also been investigated, although inhibitors in past clinical trials have induced severe side effects (Mendiola-Precoma et al. 2016). Furthermore, inhibitors of Aβ aggregation, sequesters of Aβ monomers and biological products involving microglia-mediated clearance of amyloid have reached clinical trials, as reviewed extensively (Mendiola-Precoma et al. 2016).

As mentioned earlier, non-pharmacological treatments to prevent AD are also being investigated. Lifestyle strategies including exercise, cognitive stimulation, restriction of calories and increased socialisation, as well as diet strategies including

vitamin and probiotic supplementation, restricted alcohol consumption (excluding low levels of red wine consumption), consumption of flavonoids, alkaloids, or terpenoids and inclusion of the Mediterranean and Asiatic diets, have been thought to be beneficial in preventing AD, especially in combination (Mendiola-Precoma et al. 2016). Randomised controlled trials investigating exercise and cognitive stimulation have especially shown that these lifestyle modifying strategies improve cognition, but larger trials are still required and are especially lacking for diet related strategies, as reviewed (Alzheimer's Association 2018; Mendiola-Precoma et al. 2016).

1.2 Transgenic mouse models of AD

Numerous *in vitro* and *in vivo* models of AD exist, each with merits and disadvantages in studying the disease and the evaluation of new therapeutic targets. Of importance to this study are transgenic murine models of AD, which demonstrate genetic, pathological and/or symptomatic changes relevant to AD, enabling their use as models in the study of the aetiology, pathophysiology, diagnosis and treatment of the disease (Elder, Gama Sosa & De Gasperi 2010). Although the majority of AD patients develop SAD and therefore do not carry mutations in *APP*, *PS1* or *PS2*, most transgenic murine models of AD actually model FAD, due to the ability to recapitulate amyloid pathology through the use of dominant mutations identified from FAD (Jankowsky & Zheng 2017). Therefore, single and double transgene expression of differing FAD-associated mutations in *APP*, *PS1* and *PS2* genes form the basis of the generation of the majority of transgenic mouse models for AD, which can then be used to assess amyloid pathology, which is relevant for both SAD and FAD. Furthermore, modelling SAD is challenging due to the sporadic nature of the disease, limiting most animal models of AD to transgenic murine models of FAD (Jankowsky & Zheng

2017). Mutations in the *MAPT* gene are also utilised to generate transgenic models of dementia to assess tau pathology. Most often, behavioural tests assessing social behaviours, short-term and long-term memory including recognition memory and spatial learning and memory, associative learning, anxiety, exploration, locomotion and sensorimotor gating are employed to assess AD-relevant behaviours in these models. Neuropathological analyses are also used to investigate molecular changes in the brain that are relevant to the mechanisms involved in AD.

1.2.1 *APP*$_{Swe}$*/PS1ΔE9* double transgenic mouse

Of the transgenic mice that have been generated to model FAD, the double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) mouse model was of particular interest for this thesis. Dr. David Borchelt from the University of Florida was the first to generate *APPxPS1* mice, which carry two FAD-associated mutations; the chimeric mouse/human *APP* gene with Swedish mutation (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and the mutant human *PS1* gene with exon 9 deletion (*PS1/ΔE9* (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b)). Both transgenes were co-injected at a single locus on chromosome 9 and are controlled by the mouse prion promoter, which directs transgene expression mostly to CNS neurons. This model was generated on a mixed congenic C57BL/6JxC3H/HeJ background and is maintained as a hemizygote. The *APP*$_{Swe}$ mutation was introduced by replacing the mouse DNA sequence encoding for three amino acid residues within the Aβ domain with the human sequence, which was then modified to encode the human Swedish mutation. The inserted human *PS1/ΔE9* mutation expressed in high levels displaces detectable endogenous mouse protein. Together, these two transgenes elevate the amount of Aβ produced by favouring the amyloidogenic pathway, and as a double transgene model exhibit accelerated amyloid pathology in comparison to

single transgene mouse models (Borchelt et al. 1997; Jankowsky et al. 2004a; Jankowsky et al. 2004b). Further, it is suggested that the *PS1/ΔE9* mutation causes the preferential cleavage of *APP* C99 at residue 42, causing the specific elevation of $A\beta_{42}$ levels and unaltered $A\beta_{40}$ levels (Jankowsky et al. 2004a).

As a result of the combination of the APP_{Swe} and *PS1/ΔE9* transgenes, the *APPxPS1* model is considered a particularly aggressive model of amyloid pathology (Garcia-Alloza et al. 2006), and is commonly used as a model of AD, or more specifically, of FAD. This model exhibits AD-like amyloid pathology, with Aβ plaques appearing as early as 4 to 6 months and accumulating with age (Garcia-Alloza et al. 2006; Hamilton & Holscher 2012; Jankowsky et al. 2004b; Ruan et al. 2009; Savonenko et al. 2005). *APPxPS1* mice exhibit higher levels of soluble $A\beta_{40}$ and $A\beta_{42}$ compared to control mice. The levels of these soluble peptides increase with age, with female *APPxPS1* mice showing significantly higher levels of soluble $A\beta_{40}$ and $A\beta_{42}$ compared to male *APPxPS1* mice (Wang et al. 2003). Furthermore, activated microglia and astrocytes are evident in both the hippocampus and neocortex of *APPxPS1* mice beginning at 4 months of age (microglia), and beginning at 6 months of age (astrocytes), and are found in close association with Aβ plaques (Ruan et al. 2009).

APPxPS1 mice exhibit a range of behaviours relevant to the study of AD, many of which have been shown to be associated with the pathological changes seen in the brains of the transgenic mouse model. For example, *APPxPS1* mice have been shown to exhibit spatial learning and memory deficits in the Morris water maze (MWM) and long-term contextual memory deficits in the step down passive avoidance test, which are correlated with hippocampal and cortical levels of soluble $A\beta_{40}$ and $A\beta_{42}$ (Savonenko et al. 2005; Zhang et al. 2011). Other studies have also demonstrated that

the *APPxPS1* mice have impairments in spatial learning, spatial task acquisition and spatial memory in paradigms other than the MWM, including the hidden target version of the Barnes maze and in the cheeseboard (CB) paradigm, resembling the symptoms of spatial disorientation seen in some patients with AD (Cheng et al. 2014b; O'Leary & Brown 2009). *APPxPS1* mice also exhibit task-dependent hyper-locomotion and anxiolytic-like phenotypes in the light dark (LD) test at 7 months of age, reminiscent of the restlessness and agitation seen in AD patients (Cheng et al. 2013; Cheng et al. 2014b). Some studies also report disinhibitory-like behaviours in the elevated plus maze (EPM), and social and object recognition impairments in the social preference test and novel object recognition task (NORT; Cheng et al. 2013; Cheng et al. 2014a; Cheng et al. 2014c; Lalonde, Kim & Fukuchi 2004).

To be useful as an animal model of human disease, a transgenic mouse model must display all of the following for the disease in question; face validity, construct validity and predictive validity. Face validity refers to the model possessing the essential pathological, physiological and behavioural features or symptoms relevant to the disease (Elder, Gama Sosa & De Gasperi 2010; Nestler & Hyman 2010). For example, the *APPxPS1* model displays the AD-relevant pathological hallmarks including amyloid plaques and NFT, and AD-relevant cognitive and motor impairments including spatial disorientation and recognition impairments. Construct validity refers to the similarity between human and mouse disease origin, i.e. the mouse model is to mimic the underlying cause of the disease (or aspects thereof; van der Staay, Arndt and Nordquist (2009). For example, as a model for FAD, *APPxPS1* mice are generated through genetic techniques that result in mutations in the genes *APP* and *PS1*, which are two of the three major mutations associated with the cause of FAD. Finally, predictive validity suggests that the model will respond to pharmacological therapy in

a similar manner to humans with the disease (Nestler & Hyman 2010). Memantine, a common therapy for AD, has been found to reduce the cortical levels of soluble $A\beta_{42}$ and increase the ratio of $A\beta_{40}$ /$A\beta_{42}$ in the brains of *APPxPS1* mice (Alley et al. 2010).

1.3 Medicinal Cannabis

The plants *Cannabis sativa* and *Cannabis indica* are the two most common species found in the drug cannabis (marijuana), which has a long history of human use (Lim, See & Lee 2017). Cannabis has long been known for its fibrous, psychotropic and medicinal properties and has been utilised for rope, food, recreation, religious rituals, and in medicine. Numerous cultures have recorded the medical use of cannabis for a range of ailments including pain, muscle spasms and malaria, starting with the Chinese as far back 2900BC, where cannabis is listed in the father of Chinese medicine, Emperor Shen Nung's, pharmacopoeia (Hill et al. 2017).

While the medical properties of cannabis have long been known to ancient civilizations, the introduction of medicinal cannabis to Western medicine has been only recent. Irish doctor Sir William Brooke O'Shaughnessy discovered that cannabis could treat nausea and pain in cholera patients and reported that cannabis stopped convulsions in a child while studying in India in the 1830s (reviewed in Zuardi (2006)). By the 1890s, cannabis extracts were sold in pharmacies across Europe and the United States of America as a therapy for an assortment of ailments, with over 100 publications printed on medicinal cannabis (reviewed in Hill et al. (2017)). The 1900s saw a decline in the use of medicinal cannabis due to legal restrictions and issues obtaining plants of consistent potency. However, the isolation and identification of the chemical structures of Δ_9-tetrahydrocannabinol (THC) and cannabidiol (CBD), two of the major phytocannabinoids found in the plant, and the identification of cannabinoid

receptors in the brain and of an endogenous cannabinoid system in the latter half of the 20th century sparked an increase in scientific interest for cannabis (Zuardi 2006). Since, the effectiveness and safety of medicinal cannabis has become a focus of scientific research and medicinal cannabis use is on the rise in Australia today. However, while there is copious anecdotal evidence for the efficacy of cannabis as a therapy for an array of conditions, the scientific evidence is still limited due to the historical difficulty in obtaining quality plant material owing to criminalisation of the plant in many countries.

The cannabis plant, which originated in Asia and has since spread globally, has a controversial history with legal, ethical and social implications of cannabis use restricting the scientific research of the drug as a therapeutic up until recent times. Most significant are the policies enacted in the United States of America, including the Marijuana Tax Act of 1937 and the Controlled Substances Act of 1970, which restricted and later prohibited cannabis. The criminalisation and listing of cannabis as a Schedule I drug (considered to have a high potential for abuse and no medical properties) has complicated the research of medicinal cannabis further, as procurement of cannabis for academic purposes was limited (Bridgeman & Abazia 2017). In more recent times, the legalisation of medicinal cannabis has opened the doors to civilian use of the medicine and importantly, lesser restrictions on research. Canada legalised medicinal cannabis in 2001 and recently legalised recreational cannabis and is at the forefront of research, with back logs of licence applications by researchers demonstrating the extent of research in the country (Shir & Häuser 2019; Wadman 2019). Legalisation of medicinal cannabis in individual US states (California in 1996) and in other countries such Australia, has also been important in the research of medicinal cannabis (Bridgeman & Abazia 2017).

Today, medicinal cannabis is a term applied to whole plant material, cannabis extracts, purified plant constituents (e.g. the cannabinoids THC and CBD), or particular derivatives, which are used to treat diseases and relieve disease symptoms (Lim, See & Lee 2017). Recent legalisation of medicinal cannabis has given rise to companies selling whole plant material and extracts, labelled as medicinal cannabis, as well as companies more interested in developing pharmaceutical-derived cannabis products, forming a commercial medicinal cannabis market. Several companies have produced multiple drugs derived from cannabis, including Sativex® (a 1:1 CBD:THC drug marketed by GW Pharma), Epidiolex® (liquid extract of CBD marketed GW Pharma), and synthetic THC products dronabinol (marketed as Marinol® by AbbVie) and nabilone (marketed as Cesamet® by Valeant Pharmaceuticals International Inc), as reviewed previously (Mintz 2015). However, there are also numerous other analogues of cannabinoids and endocannabinoids that have been designed to be more enantiomerically pure, more metabolically stable and more potent than naturally derived constituents, including "HU-211" and "CT3" (Di Marzo, Bifulco & De Petrocellis 2004). Aside from synthetic analogues, pure constituents of cannabis are also used in clinical trials. For example, the purified cannabis constituent CBD has garnered much attention in the scientific community recently and is central to my *Master of Research* project. However, before a more in-depth examination of CBD carried out in section 1.3.2, a brief insight into the endocannabinoid system (ECS) must be considered.

1.3.1 The endocannabinoid system (ECS)

The ECS is the endogenous cannabinoid system involved in numerous basic functions of the human body including learning, memory, neuronal development, pain sensation, inflammation, appetite, digestion, thermogenesis, the sleep-wake cycle,

mood and addiction. Cannabinoid receptors, endogenous cannabinoids (endocannabinoids) and various enzymes involved in the biosynthesis or degradation of the endocannabinoids make up the ECS. (Aizpurua-Olaizola et al. 2017; Di Marzo, Bifulco & De Petrocellis 2004).

Cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2) are the two main G-protein-coupled receptors of the ECS, to which cannabinoids bind to affect their function. CB_1 are highly abundant and found mostly in neurons, glial cells and many peripheral cells. They are highly expressed in the basal ganglia, cerebellum, hippocampus and the dorsal primary afferent spinal-cord regions, accounting for the effect of cannabis on motor coordination, learning and memory and pain regulation. Furthermore, CB_1 are found at low concentrations in the brain stem, which accounts for the lack of acute fatalities (Baker et al. 2003). CB_2 are found in immune cells such as B and T lymphocytes, macrophages and microglia, suggesting that the ECS has a role in immunomodulation and mediation of neuroinflammation, as reviewed (Stella 2010). Activation of CB_1 is responsible for the psychoactivity of cannabinoids, while activation of CB_2 is not (Ramírez et al. 2005).

The two most well-known endocannabinoids are arachidonoylethanolamide (anandamide) and 2-aracidonoylglycerol (2-AG), which are biosynthesised when required by phosphodiesterase enzymes from membrane-associated fatty-acid precursors. These endocannabinoids then bind to and stimulate CB_1 and CB_2 second messenger pathways, mainly to regulate neurotransmission, and are then degraded through reuptake and hydrolytically cleaved by fatty acid amide hydrolase (FAAH; metabolises anandamide) or monoacylglycerol lipase (metabolises 2-AG). Anandamide and 2-AG are degraded to compounds that do not have cannabinoid receptor binding activity, such as arachidonic acid (Baker et al. 2003). The most well-

known exogenous cannabinoids include the plant-derived phytocannabinoids THC and CBD and the synthetic cannabinoids CP 55,940 and WIN 55,212-2 (Karl, Garner & Cheng 2017). These exogenous cannabinoids can bind to cannabinoid receptors and therefore stimulate the ECS, presenting a potential therapeutic opportunity discussed further below.

The characteristic "on-demand" biosynthesis of endocannabinoids gives the ECS an adaptive response to changing conditions and the ability to restore homeostasis. However, in diseases such as AD, the ECS has been shown to be altered and associated with disease symptoms and progression, with post-mortem, *in vitro* and *in vivo* analyses having found dysregulated expression of some components of the ECS (Aizpurua-Olaizola et al. 2017). For example, a recent post-mortem study found that midfrontal and temporal cortex tissue from AD patients had significantly lower levels of anandamide compared to controls, which was inversely correlated to $A\beta_{42}$ levels (but not $A\beta_{40}$ or plaque load) and cognitive symptoms (Jung et al. 2012). Others have found that FAAH and CB_2 were upregulated in microglia found proximal to plaque associated areas of post-mortem AD hippocampus tissue, with CB_1 levels unchanged compared to controls (Benito et al. 2003; Mulder et al. 2011). Furthermore, *in vitro* and *in vivo* models provide additional evidence that the ECS is disrupted in AD. For example, in a microglial model, stimulation of CB_2 with synthetic cannabinoids resulted in enhanced Aβ phagocytosis, and treatment with particular cannabinoids blocked Aβ induced activation (Ramírez et al. 2005). Further, stimulation of CB_2 with specific CB_2 agonists is capable of inducing the removal of Aβ from human frozen tissue sections by macrophages (stimulation with JWH-015; Tolón et al. (2009)), and of inducing cognitive impairment and a reduction in microglial activity in *APPxPS1* mice (stimulation with JWH-033; Aso et al. (2013)). Other studies have identified

increased levels of CB_2 and 2-AG, and decreased levels of CB_1 and anandamide, in rats treated with $A\beta_{42}$ (Esposito et al. 2007a). Furthermore, the expression of CB_1 has been found to be upregulated in the prefrontal cortex (PFC), amygdala and dorsal hippocampus, and downregulated in the ventral hippocampus of 6 and 12-month-old 3xTg-AD mice (a triple transgenic mouse model of AD with mutations in *APP*, *PS1* and *MAPT*; Bedse et al. (2014)). Also, genetic deletion of CB_2 in *APPxPS1* mice results in reduced levels of microglia, infiltrating macrophages, pro-inflammatory chemokines and cytokines, and a reduction in soluble Aβ (Schmöle et al. 2015), whereas genetic deletion of CB_1 in *APPxPS1* mice accelerates memory impairment in the two-object recognition test (Aso, Andrés-Benito & Ferrer 2018). Thus, the involvement of the ECS in AD is complex and the ECS has been recognised as a potential therapeutic target for AD interventions.

1.3.2 Cannabidiol (CBD)

CBD is the main non-toxic (non-"high" producing) phytocannabinoid of the plant *C. sativa* and is known to possess antioxidant, anti-apoptotic, neuroprotective, immunosuppressive and anti-inflammatory properties. Accordingly, numerous studies demonstrate a variety of pharmacological properties of CBD, including anti-convulsive, anti-anxiety, anti-psychotic, anti-emetic, anti-inflammatory and anti-rheumatoid arthritis (reviewed in Karl, Garner and Cheng (2017)). Also, CBD has very low toxicity and high lipophilicity, meaning it is readily able to cross the blood-brain barrier. CBD may have properties enabling it to reduce amyloid and tau pathologies, and unlike other cannabinoids does not impair cognition (Karl, Garner & Cheng 2017). These properties suggest that CBD may be relevant for the treatment of neurodegenerative diseases, especially AD.

CBD has low affinity for cannabinoid receptors, especially compared to other cannabinoids such as THC, and likely exerts its effect on the ECS as a non-competitive antagonist of CB_1 and CB_2 agonists. This may in part explain the anti-inflammatory effect of CBD, as inverse agonism of CB_2 inhibits immune cell migration (Karl, Garner & Cheng 2017). CBD may also exert its effect through activation of various other targets such as the G-protein coupled receptor 55, transient receptor potential vanilloid type 1 and the serotonin 1A receptor (Hartmann et al. 2019), and has been shown to inhibit anandamide uptake and hydrolysis, and blunts fatty acid amide hydrolysis, each of which help explain the effect of CBD on the ECS as this results in an increase in anandamide, allowing CBD to indirectly stimulate cannabinoid receptors (Hartmann et al. 2019). CBD has also demonstrated the abilities to protect against neurotoxicity via inhibition of uptake of an adenosine transporter (Carrier, Auchampach & Hillard 2006) and via reduction of glutamate toxicity (Hampson et al. 1998), and increases hippocampal neurogenesis *in vivo* (Wolf et al. 2010).

It has been observed that cannabinoids, including CBD, produce biphasic dose response curves in relation to anxiety, feeding behaviour, wakefulness, locomotion, exploration and ACh release, among other effects. Evidence suggests that rather than producing dose-dependent responses, relative high and low dosages of a cannabinoid can i) produce the same effect, ii) produce greater effects than medium dosages, or vice versa, or iii) have the opposite effect of each other (Rey et al. 2012; Tzavara, Wade & Nomikos 2003). For example, preclinical studies have demonstrated that CBD produces inverted U-shaped dose-response curves in numerous strains of rats, mice and zebrafish in paradigms including the elevated plus maze, prepulse inhibition, and memory related tasks, as reviewed (Rey et al. 2012; Zuardi et al. 2017). The *in vivo* anxiolytic, anti-inflammatory action and post-ischemia neuroprotection of CBD

has also been demonstrated to have bell-shaped dose-dependency. In human volunteers with insomnia, relatively high doses of CBD increases sleep duration, while lower doses of CBD increase alertness. CBD also produces biphasic dose responses in the suppression of lithium induced vomiting (Zuardi 2008), and the bell-shaped dose-dependency of CBD's anxiolytic effect *in vivo* has been replicated in human studies of anxiety in public speaking (Zuardi et al. 2017). It has been suggested that the biphasic nature of cannabinoids is related to distinct sub-populations of cannabinoid receptors. For example, low doses of the WIN55,212-2 induces a transient stimulation of hippocampal ACh efflux *in vivo*, while a high dose results in a prolonged inhibition of ACh efflux. There is evidence that the difference in effects of the synthetic cannabinoid was due to a differential sensitivity of distinct subpopulations of CB_1 that are linked to differing neurotransmitter systems that have opposing effects on ACh release (Tzavara, Wade & Nomikos 2003). Furthermore, another study demonstrated that a low dosage of CB_1 agonist CP 55,940 has anxiolytic properties while a high dose has anxiogenic properties. The authors of this study also suggested that the biphasic effect on anxiety was due to distinct subpopulations of CB_1 that had differing sensitivities and were linked to either glutamatergic terminals or GABAergic terminals (Rey et al. 2012). Thus, since CBD, as other cannabinoids, exhibits bell-shaped dose response curves, it is pivotal to investigate a range of dosages to determine the window of therapeutic effectiveness of the drug.

1.3.3 Evidence for CBD as a therapeutic of AD

Various properties of CBD are relevant in the fight against numerous pathological symptoms of AD, including the properties of neuroprotection, anti-inflammation and antioxidant effects. As such, CBD has shown potential as a therapeutic for AD in preclinical studies. Numerous *in vitro* studies utilising rat PC12 neuronal cells

stimulated with Aβ have shown that CBD acts against Aβ-induced toxicity in various ways. For example, CBD is able to inhibit tau hyperphosphorylation in a dose-dependent manner in these cells, and is associated with a reduction in the phosphorylated glycogen synthase kinase 3-β, which is responsible for NFT formation in AD (Esposito et al. 2006a). Similarly, other studies show that CBD increases cell survival and reduces Aβ-induced lipid peroxidation, ROS production (Iuvone et al. 2004), and attenuates NO production via inhibition of phosphorylated p38 mitogen-activated protein kinase and transcription factor nuclear factor-κB (Esposito et al. 2006b). CBD has also demonstrated the ability to counteract the elevation of APP expression in transfected human neuroblastoma cells by inducing ubiquitination of APP through activation of peroxisome proliferator-activated receptor-γ (PPARγ), which is paralleled by a reduction of Aβ peptide expression and increased cell survival (Scuderi, Steardo & Esposito 2014).

CBD has also shown potential *in vivo* as a therapeutic for AD. In a pharmacological mouse model of AD-related neuroinflammation generated by hippocampal injection of Aβ (of lengths one to 42 amino acids), the anti-inflammatory properties of CBD were shown to attenuate Aβ-evoked neuroinflammation in a dose dependant manner (Esposito et al. 2007b). In a later study using a rat model this effect was identified to be mediated via PPARγ (Esposito et al. 2011)), which has been shown to be elevated in AD patients (de la Monte & Wands 2006). CBD treatment is also able to prevent an Aβ-induced learning deficit of a pharmacological mouse model of AD in the MWM (Martín-Moreno et al. 2011). Important for this *Masters of Research* study is the finding that a moderate dosage of CBD, 20 mg/kg bodyweight, has been shown to reverse social recognition and novel objection recognition deficits in 6-month-old *APPxPS1* mice when delivered chronically post-onset of disease-relevant symptoms

(Cheng et al. 2014a). This dosage also prevented the development of a social recognition deficit in the *APPxPS1* model when delivered for 8 months prior to the onset of disease symptoms (Cheng et al. 2014c). Similarly, botanical extracts of CBD-rich cannabis given to *APPxPS1* mice at a dosage of 0.75 mg/kg of CBD were found to increase the recognition index of mice in the two-object recognition test when chronically administered during the early symptomatic stage of AD (Aso, Andrés-Benito & Ferrer 2016).

1.4 Aims

1.4.1 Rationale and hypothesis

There is currently no cure for AD. Without a significant medical breakthrough, it is expected that AD and dementia will cost Australia upwards of $1 trillion by 2056 and will have significant global impacts also. Development of an effective therapeutic agent is urgent and will benefit not only patients but also their families, caregivers, and health systems. The current project contributes to a field of research seeking a treatment for AD through a preclinical investigation of the therapeutic potential of low dose CBD in an animal model relevant for AD, and may guide the development of human clinical trials (e.g. in advising drug dose, route of administration, primary treatment measures, potential side effects etc.), and may eventually lead towards an approved drug for the disease. Further, this project will contribute new insights into CBD biology and function for the field of behavioural neuroscience, with the findings more specifically adding knowledge about the effects of CBD in the *APPxPS1* mouse model of AD and the effects of chronic CBD administration in an ageing organism. Therefore, in endeavouring to determine the efficacy of CBD as a treatment for

dementia, the current project will contribute to the preclinical research effort necessary to move toward approval of a treatment for AD to reduce the burden of this disease.

There is preclinical evidence that 20 mg/kg body weight CBD is able to reverse and prevent signs of AD in the *APPxPS1* model, yet a low dosage of 5 mg/kg body weight CBD is yet to be studied in the *APPxPS1* model. As CBD shows biphasic properties, it is hypothesized that chronic treatment of 5 mg/kg body weight might show a similar capability to reverse cognitive impairments in the *APPxPS1* mouse model as did 20 mg/kg body weight CBD, rather than exhibiting dose dependent effects. Furthermore, due to the biphasic dose response curve of CBD, there is the potential that the chosen low dosage of CBD may have the opposite effect to that of 20 mg/kg bodyweight CBD, and therefore is necessary to investigate this low dosage. It is important to investigate the potential of CBD at a range of dosages, with a low dosage of CBD having clinical importance regarding potential minimization of the cost of therapy, if CBD were considered as a therapeutic.

1.4.2 Major aim

To determine if a chronic administration regime of 5 mg/kg bodyweight CBD can reverse cognitive and motor impairments in the female *APPxPS1* transgenic mouse model of AD at 12 months of age.

Chapter 2: Materials and Methods

2.1 Animals

12-month-old female double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) mice were used in this study to model AD, specifically FAD. This model was chosen as *APPxPS1* mice replicate the most relevant features of AD, including cognitive deficits and pathological features such as Aβ deposition and oxidative stress damage (Aso et al. 2015). Female mice were chosen due to availability and also as female *APPxPS1* mice display higher levels of soluble $A\beta_{40}$ and $A\beta_{42}$ compared to males as mentioned previously. At 12 months of age the mice are considered to be in an advanced stages of the symptomatic phase of AD (Aso, Andrés-Benito & Ferrer 2016). This model bears the Swedish mutation of the chimeric mouse/human *APP*, and the exon-9-deleted *PS1* (*PS1ΔE9*) mutation on a mixed C57BL/6JxC3H/HeJ background (Borchelt et al. 1997; Jankowsky et al. 2004b), as previously mentioned. Female *APPxPS1* mice (n = 22) and their non-transgenic wild type-like littermates (WT: n = 28) were 361 ± 8 days old at the onset of the study, with a total of three cohorts of mice being used. Mice were bred at Australian BioResources (ABR: Moss Vale, NSW Australia) where they were grouped housed in individually ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) under a 12/12 h light/dark cycle with a dawn/dusk simulation. Mice were transported to the Western Sydney University animal facility (School of Medicine, Campbelltown, Australia) once they had reached adulthood where littermates were group housed (2-4 mice per cage). Two weeks prior to the start of any experiments, cages containing four mice were separated, ensuring group housing of 2-3 mice per cage for the duration of the experimental period. Mice were housed in high temperature polysulfone filter top cages (1284L: Tecniplast, Rydalmere Australia) and provided with food (Rat & Mouse Pellets: Gordon's

Specialty Stockfeeds Pty Ltd., NSW, Australia) and water *ab libitum* unless otherwise described. Corn-cob bedding (PuraCob premium: Able Scientific, Perth, Australia) as well as crinkle paper (Crink-l'Nest, Kraft) and tissue for nesting were used but no enriching structures. Cages were changed fortnightly. Standard laboratory conditions applied with a 12/12 h light/dark cycle (light phase beginning 0900 with white light at an illumination of 124 lux, and dark phase beginning 2100 with red light at an illumination of less than 2 lux). Temperature and relative humidity were automatically controlled between 20-22 °C and 40-60%, respectively. All procedures were approved by the Western Sydney University Animal Care and Ethics Committee (#A12905) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.2 Drug preparation and administration

A preparation of powdered cannabidiol (CAS: 13956-29-1; THC Pharma GmbH, Frankfurt/Main, Germany) dissolved to a concentration of 0.5 mg/mL in equal parts of Tween80 (Sigma-Aldrich Co., St Louis, USA) and 100% ethanol, and diluted in 0.9% saline, to a ratio by volume of 1:1:18 ethanol:Tween80:saline was used to prepare the CBD treatment solution. A similar solution without the addition of powdered cannabidiol (1:1:18 ethanol:Tween80:saline) was used as the vehicle.

CBD treatment and vehicle preparations were made up weekly. Firstly, all falcon tubes for storage and preparation of the CBD treatment (but not vehicle) were siliconized using a solution of a chlorinated organopolysiloxane in heptane (Sigmacote; Sigma-Aldrich Co., St Louis, USA). A thin layer of Sigmacote was coated onto the inner surface of the falcon tube and allowed to dry to prevent surface adsorption of CBD to the plastic. Then, an appropriate weight of powdered CBD was

measured using a fine precision balance and dissolved in the appropriate volume of the chosen solvent, 100% ethanol. This was vortexed in the siliconised falcon tube for approximately 20 s until fully dissolved. An appropriate volume of the surfactant Tween80 was added to the solution, which was then vortexed for another 20 s. Finally, 0.9% saline was added to the solution to a final ratio by volume of 1:1:18 ethanol:Tween80:saline. This was vortexed for 20 s until completely emulsified.

Both ethanol and Tween80 were required to dissolve and facilitate the emulsification of CBD in saline, as the hydrophobic properties of CBD mean that it does not readily dissolve in saline alone. While 10% ethanol and 10% Tween80 have been used as drug vehicles in previous CBD studies, this study chose to limit the negative effects that these reagents potentially have on mice by choosing a lower percentage of ethanol and Tween80 in the final stock preparations, based on previous studies that have had success using the stock volume ratio of 5% ethanol and 5% Tween80 (Cheng et al. 2014a). Consideration of the final amount of solvent in the vehicle preparation is important considering that ethanol and Tween80 may be toxic and can cause altered behavioural effects in mice, such as increased or decreased locomotor activity (Castro et al. 1995), when used as drug vehicles at high (16-32%) percentages. However, it has been shown that a lower percentage of 5% ethanol/Tween80 does not alter locomotor activity (though a combination of the two may; Castro et al. (1995)).

Half of the weekly CBD batch was refrigerated at 4 °C and used immediately for four days, while the other half was frozen at -18 °C for up to three days and defrosted and refrigerated for use over the next three days. CBD storage vessels were covered in aluminium foil to prevent light exposure to the solution to ensure the CBD remained stable, as CBD, like other cannabinoids, is light sensitive and should be stored in

darkness (Fairbairn, Liebmann & Rowan 1976). Enough vehicle was prepared to last seven days and stored at 4 °C.

Mice were quasi-randomly assigned to either treatment or control groups, and it was ensured that treatment assignment was counterbalanced across genotype and within cages for each cohort. Four experimental groups were set up with the number of animals in each group as follows: WT treated with vehicle (WT-VEH), n = 15; WT treated with CBD (WT-CBD), n = 13; *APPxPS1* treated with vehicle (*APPxPS1*-VEH), n = 10; and *APPxPS1* mice treated with CBD (*APPxPS1*-CBD), n = 12. At approximately 12 months of age, mice were treated daily with CBD or vehicle for three weeks prior to the start of the experiments and daily treatment continued throughout the behavioural assessment. The prepared CBD or vehicle solutions were administered by intraperitoneal (i.p.) injection at an injection volume of 10 mL/kg body weight, utilising a CBD dosage of 5 mg/kg body weight. The route of administration was chosen to be via i.p. injections, rather than other modes of administration such as oral or intravenous routes, due to the 100% bioavailability of this route and the chronic nature of the study (Machholz et al. 2012). Furthermore, i.p. injections are more established and in line with other studies investigating the potential of chronic administration of CBD as a therapeutic in *APPxPS1* mice (Cheng et al. 2014a). Treatment was always administered in the afternoon to avoid acute effects of the injections modifying the behavioural performance of the mice during the experimental test period. The site of injection was alternated daily between left and right lower abdomen. Body weight was monitored weekly.

2.3 Behavioural test battery

Mice performed a battery of behavioural paradigms described below to assess anxiety, motor function, cognition, and sensorimotor gating. All of these behavioural domains have been found to be affected in dementia or AD-relevant mouse models. In line with previous studies conducted in our laboratory (Cheng et al. 2014a), all experiments were performed during the first 5 h of the light phase to reduce the effects of the circadian rhythm on mice performance (i.e. to avoid the less active period of the light phase; Grech et al. (2019)), and a 48 h inter-test interval applied for all behavioural testing to minimise the effect of repeated testing and to allow mice to rest between tests (with the exception of low-impact motor function tests, which were performed over three consecutive days). On the day of testing, mice were habituated to the test room for 30-60 min prior to testing. Mice were euthanised and tissue collected following assessment (further discussed in section 2.4). For a diagrammatic view of the study design, please see Figure 3. For an overview of test order and test age, please see Table 1.

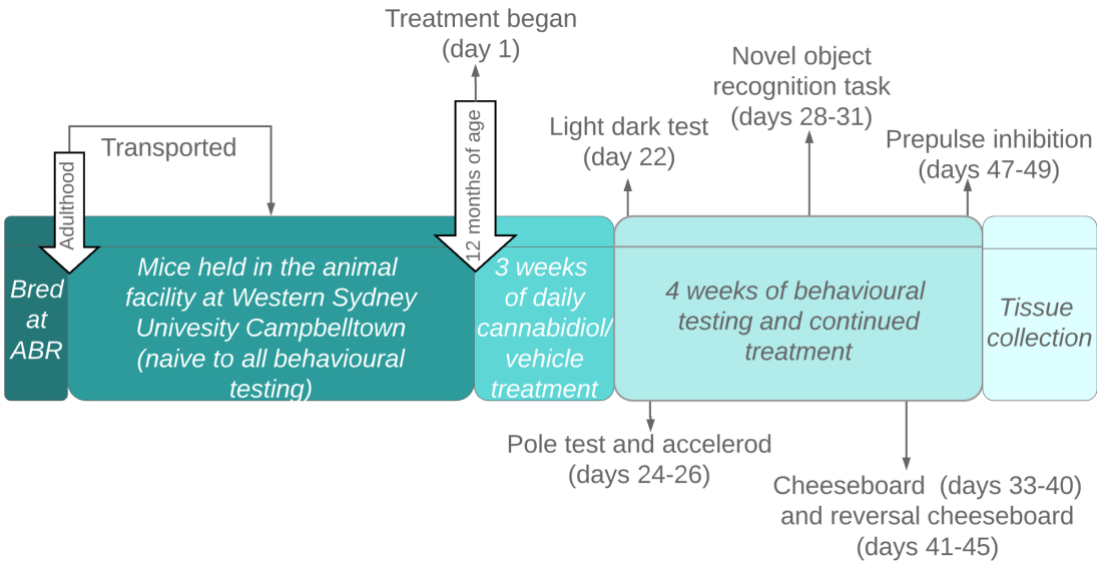

Figure 3: Outline of the study design.
Example of the days on which behavioural testing was conducted for cohort 1 (cohort 2 and 3 differed slightly). *Abbreviation*: Australian BioResources (ABR).

Table 1: Test biography.
Test order and test age [in days, on the first day of each test] of non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Ages [d] are presented as mean ± standard error of means (SEM). *Abbreviation*: Novel object recognition task (NORT). No significant differences between days of test.

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Start of CBD treatment	361 ± 2	357 ± 2	363 ± 2	360 ± 2
Light Dark	382 ± 2	378 ± 2	384 ± 2	381 ± 2
Pole Test	384 ± 2	380 ± 2	386 ± 2	383 ± 2
Accelerod	384 ± 2	380 ± 2	386 ± 2	383 ± 2
NORT	389 ± 2	385 ± 2	391 ± 2	388 ± 2
Cheeseboard	392 ± 2	388 ± 2	394 ± 2	391 ± 2
Prepulse Inhibition	406 ± 2	404 ± 2	408 ± 2	405 ± 2
Tissue Collection	409 ± 2	407 ± 2	411 ± 2	408 ± 2

2.3.1 Light dark (LD)

Mice have a natural tendency to explore novel environments while also showing aversion to brightly illuminated and exposed areas. Thus, anxiety-related behaviour (Crawley 1985) can be assessed by placing mice into the light dark test (LD). In the LD, mice are placed into a box that is divided into an enclosed dark zone, which mice show a preference for (Crawley 1985), and an exposed and illuminated light zone, which mice are inclined to explore but also apprehensive of (that is, they show state (spatio-temporal) anxiety; Ramos (2008)). The level at which a mouse explores the light zone in comparison to the dark zone is indicative of their level of anxiety, with more exploration of the light zone suggestive of reduced anxiety. The behaviours of mice in the LD can also be used to assess general activity, for example exploratory

rearing and distance travelled. Thus, the LD test not only tests for state anxiety but also assesses exploratory and locomotive behaviours.

An assessment of anxiety, exploration and locomotion was necessary for this study. Cannabinoids have been shown to instigate biphasic anxiety responses (Rey et al. 2012), and both patients with AD and the *APPxPS1* mouse model of AD show changes in anxiety (Lalonde, Kim & Fukuchi 2004) and locomotion (Alzheimer's Association 2018; Cheng et al. 2014b), necessitating the need to investigate these factors. Importantly, stress, anxiety and emotion can impact upon cognition (Brinks et al. 2007; Harrison, Hosseini & McDonald 2009), so it is essential to examine the anxiety phenotype of mice in the LD to consider its impact on other cognitive tests.

The apparatus (Figure 4) had a total test area of 43.2 cm x 43.2 cm and consisted of two equally sized zones: a black and covered (dark: illumination <20 lux) zone, and a white and uncovered (light: illumination >200 lux) zone. This was achieved by placing a black infrared transmitting plastic dark box insert (model ENV-516: MED Associates Inc., St Albans, VT, USA) into the rear half of an infrared photobeam controlled open field test chamber (model ENV-515: MED Associates Inc., St Albans, VT, USA). An opening in the centre of the dark box insert allowed passage between the two zones. After a 1 h habituation to the test room, mice were placed into the dark zone and allowed to explore the entire apparatus for 10 min. 80% ethanol was used to clean the apparatus between mice. Total distance travelled in the dark zone and total distance travelled over time were used as indicators of locomotion. Exploration was shown by the frequency of *rearing* (vertical activity). Percentage time spent and percentage distance travelled in the dark zone were calculated to identify anxiety-related behaviours.

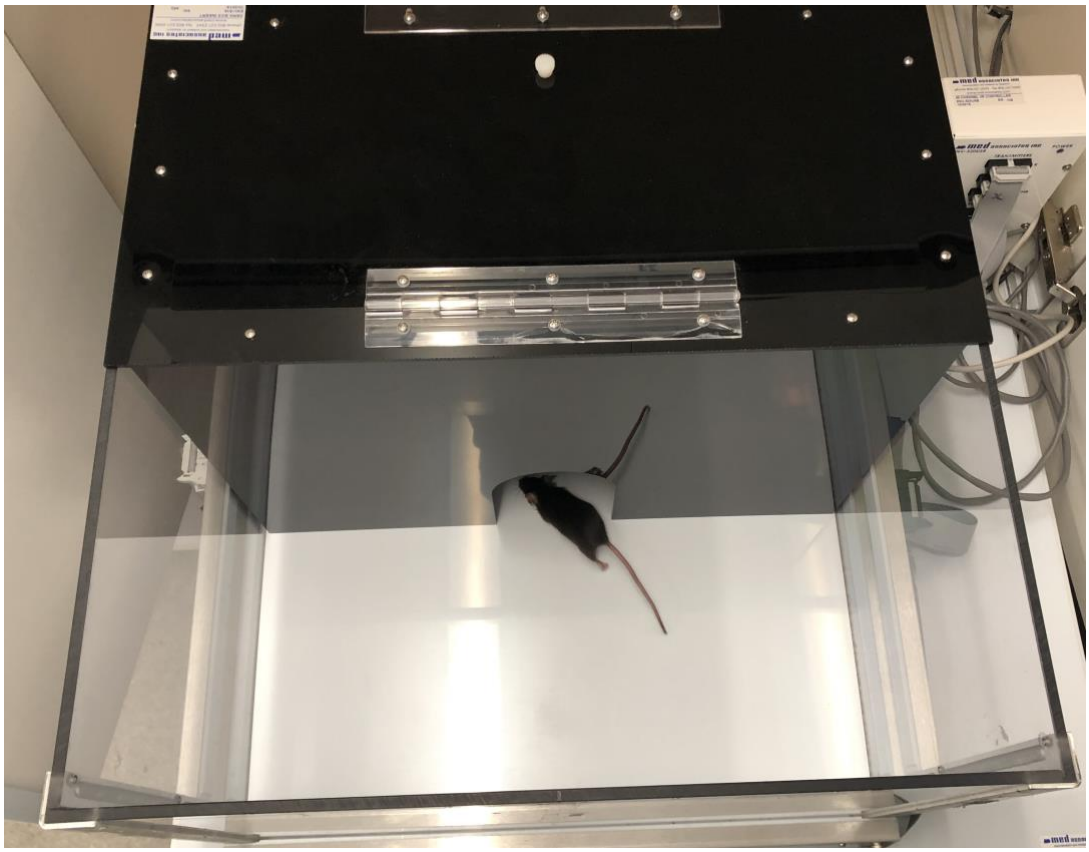

Figure 4: Light dark test apparatus.
Mice were placed into the dark zone of the apparatus at the beginning of the test through the hinged lid of the dark box insert, and allowed to explore for 10 min.

2.3.2 Pole test

Motor function can become dysfunctional in some AD patients, and the *APPxPS1* model has previously shown motor deficits at 6 months of age (Kuwabara et al. 2014), but not in other studies testing at 7 months of age (Lalonde, Kim & Fukuchi 2004); thus, this study explored the motor function of test mice.

Non-specific motor function was assessed using the vertical pole test, whereby climbing behaviour, which involves multiple aspects of motor function, is measured (Brooks & Dunnett 2009). The apparatus for the pole test (Figure 5) was a 50 cm aluminium pole of 1 cm diameter wrapped with masking tape for grip and mounted on a base platform. After a 30 min habituation to the test room, mice were placed with snouts facing upwards on the end of the pole, which was held horizontally at that stage.

Once the mice had grip, the pole was turned upright to a vertical position and the mice were allowed to turn around and climb down the pole. Performance was measured by the time taken to turn around (latency to inversion), the time taken to descend the pole once turned around (time to descend), and the total time taken to reach the platform from the beginning of the test (latency to reach the platform; cut off time of 60 s). All aforementioned behaviours were recorded manually using a stopwatch. It was also recorded as to whether the mice wrapped their tails around the pole for balance, however this was not analysed (Figure 5). This procedure was repeated a total of three times with a 30 min inter-trial interval (ITI) and the apparatus was cleaned with 80% ethanol between trials. The average of the three trials was considered for analysis.

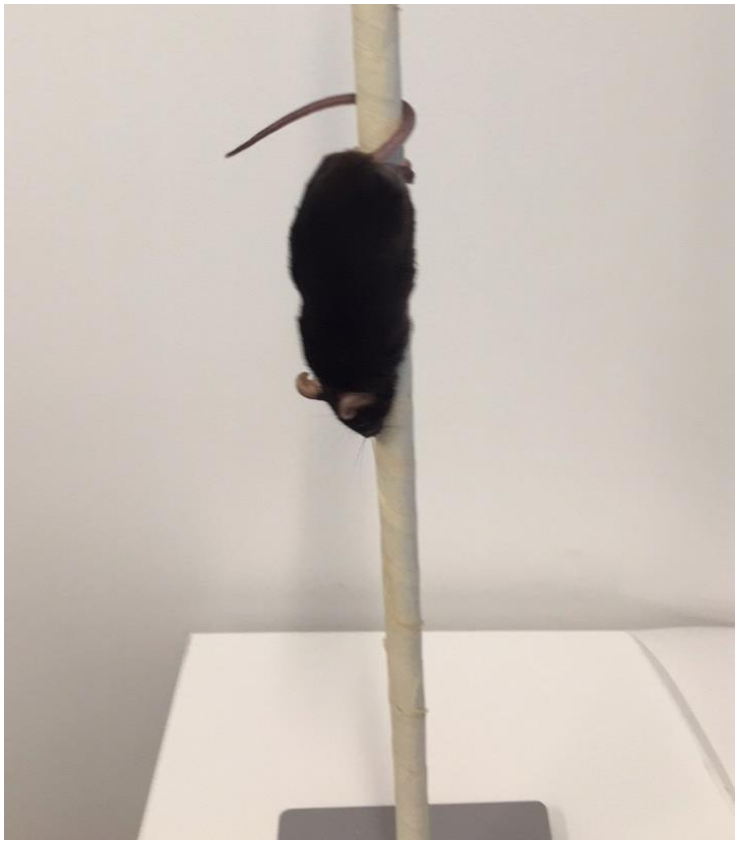

Figure 5: The pole test apparatus.
Mice were given a maximum of 60 s to climb down the pole to the platform. Tail wrapping is evident.

2.3.3 Accelerod

An accelerating rotarod paradigm was used to measure the motor coordination and balance of the test mice (Brooks & Dunnett 2009), which as previously mentioned can become impaired in AD and in the *APPxPS1* mouse model. The accelerod test was conducted over three days, with the first day occurring on the same day as the pole test; the pole test was completed first, and following a 1 h ITI, mice began training to the accelerod. The apparatus (Rota-rod model ENV 574M; MED Associates Inc, St. Albans, VT, USA; Figure 6) is a five-lane Rota-rod treadmill for mice. On the first day mice underwent training to the accelerod to ensure mice understand the task before getting tested. During the training, mice were placed on the rotating cylinder of the apparatus, facing the opposite direction of rotation, which ensured the mice walked to stay on the beam. Mice were given three x 2 min training trials with the beam rotating at a continuous speed of 12 revolutions per minute (rpm), with an ITI of approximately 20 min. Mice were replaced on the accelerod if they fell off during training. On the second and third day, during the test phase, mice were habituated to the room for 30 min, and were then placed on the beam, which was set to slowly accelerate from 4 rpm to 40 rpm over a 4.5 min period, and ended with a 30 s period at a continuous 40 rpm speed. Performance was measured as the time at which mice fall from the cylinder (latency to fall), which was automatically detected and recorded by sensors. This procedure was repeated twice per day with an ITI of 1 h (four test trials across two days). 80% ethanol was used to clean the apparatus between trials. The mean of the four trials was considered for analysis, as was the worst performing and best performing trials.

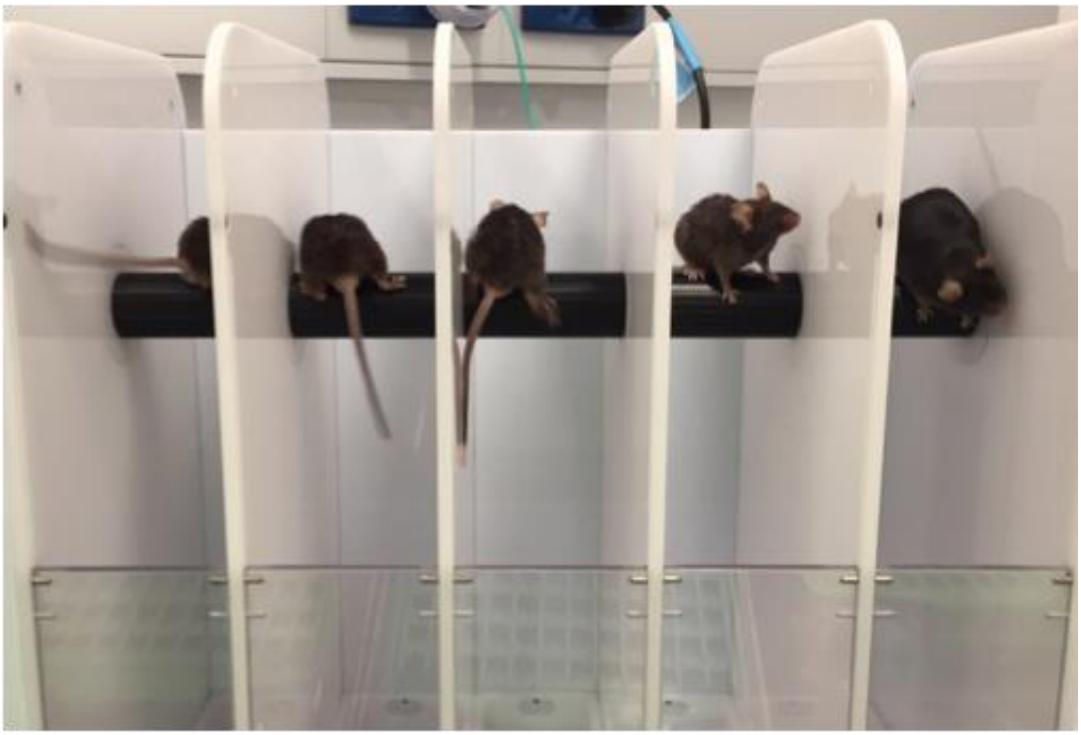

Figure 6: The accelerod apparatus.
The Rota-rod treadmill for mice consists of a rotating beam and allows for testing of up to five mice at a time.

2.3.4 Novel object recognition task (NORT)

The innate preference of a mouse for novelty, and their ability to distinguish a novel object from a familiar object (Dere, Huston & De Souza Silva 2007) is utilised in this test to determine object recognition memory, an aspect of cognition. Cognition, and especially recognition, is severely impaired in AD patients and is replicated in the *APPxPS1* model, and may be able to be rescued by CBD (Cheng et al. 2014a; Cheng et al. 2014c). Thus, object recognition memory was assessed in this study.

The apparatus for the NORT paradigm was a grey PVC arena (300 mm x 350 mm x 350 mm; Figure 7), which mice were habituated to for 10 min one day prior to testing. On the test day, 24 h later, mice were habituated to the test room for 30 min. The testing was carried out across two trials. In trial 1, the 'training' trial, two identical objects (each a stack of LEGO blocks) were placed and secured via a reusable adhesive (so that mice could not move the objects) in opposite corners of the NORT arena, and

mice were placed into one of the remaining empty corners and allowed to explore the arena and the objects for 10 min. In trial 2, the 'testing' trial, one of the now 'familiar' objects from trial 1 was replaced with a LEGO Duplo giraffe (the 'novel' object). Mice were again placed into one of the empty corners and allowed to explore arena and objects for 10 min. A 15 min ITI was used and the arena and objects were cleaned with 80% ethanol between trials to remove any scent cues. The location of the novel object (left corner or right corner) was counterbalanced across genotype. Both trials were video recorded using AnyMaze tracking software (Stoelting, Wood Dale, USA), and the time spent *nosing* the objects was later manually scored (*nosing* was determined when the animal's nose was facing the direction of the object and was sniffing at a distance of less than 5mm away). The percentage of time spent *nosing* the novel object was calculated [(novel object nosing time) / (novel + familiar object nosing time) × 100] and used as an indication of object recognition memory, also known as the recognition index. One *APPxPS1*-CBD mouse died prior to NORT testing. Of the remaining mice, one WT-VEH mouse and one *APPxPS1*-CBD mouse were excluded from analysis, as these mice did not meet the requirements of the task (a minimum of 20 s exploration during both trials), as they showed extensive *freezing* behaviour during training and/or testing. This exclusion criterion is in line with previously published studies from our lab using NORT (Cheng et al. 2014a). Extensive *freezing* was defined as immobility for greater than 90% of the trial.

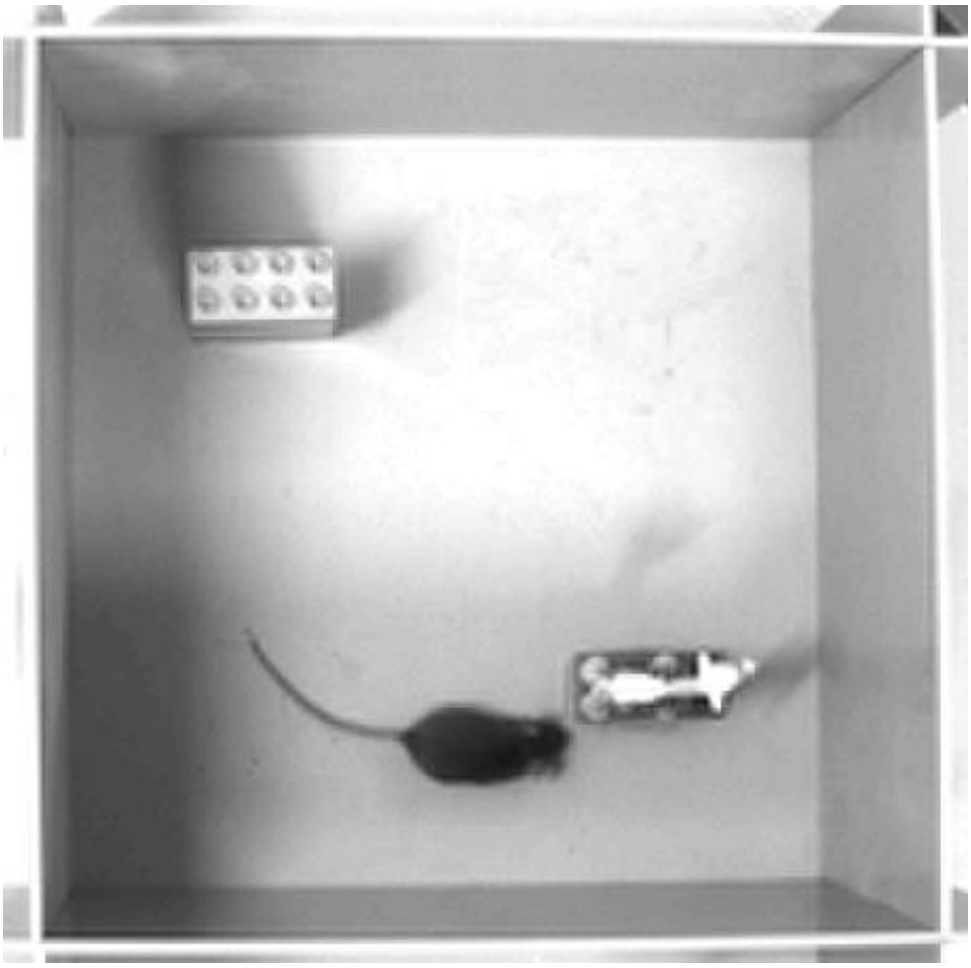

Figure 7: The novel object recognition task (NORT) paradigm; testing trial.
In the square, grey NORT arena, a stack of LEGO blocks (upper left) was used as the 'familiar' object, while a LEGO Duplo giraffe (lower right) was used as the 'novel' object in the testing trial.

2.3.5 Cheeseboard (CB)

Spatial disorientation is a significant symptom of AD patients and is replicated in the *APPxPS1* model (Cheng et al. 2014b; Reiserer et al. 2007); thus, aspects of spatial memory were assessed in this study through the cheeseboard (CB) paradigm. The CB was chosen as a less-strenuous dry-land substitute for the MWM to assess spatial learning acquisition and spatial reference memory, in line with previous studies in our lab (Cheng et al. 2014b). Unlike the MWM, the CB uses positive reinforcement (a food reward) and motivates mice to learn through hunger, rather than the motivation

of survival to learn to avoid drowning (Grech et al. 2019). This factor makes the CB a less-stressful alternative to the MWM. Furthermore, unlike rats, for which the MWM was originally designed for, mice are not natural swimmers and can show exhaustion in the MWM, as well as *floating* behaviour, thigmotaxis and hypothermia (Lopez et al. 2010); thus, employment of the more ideal CB in this study eliminates these factors.

The apparatus was a circular grey wooden board (110 cm diameter; Figure 8), with one side blank, and one side covered by 32 wells, arranged in eight equal zones containing four wells each. The board was placed in the test room and surrounded by visual external cues that were not changed during testing to enable spatial orientation. External cues were large black or white shapes, for example a circle, square or plus sign (Figure 5), on contrasting backgrounds placed no more than 30 cm from the edge of the board. The illumination of the test room was dimmed to 50 lux to ensure mice were not afraid while on the board, which was 75 cm from the ground.

Motivation to find the food reward during testing was achieved through a food deprivation schedule. Mice were food restricted, beginning in the afternoon of the day prior to habituation to the apparatus, to a maximum of 85% of their free-feeding body weight throughout the entire testing period. One hour following completion of the last trial of each daily cheeseboard task, mice were given access to food for at least 60 min and up to 120 min if required to guarantee the indicated weight loss. Food was provided *ad libitum* following the final reversal probe. The food reward was sweetened condensed milk diluted with water to a ratio of 1:4 milk:water. Mice were habituated to the food reward immediately following the final trial (and prior to feeding for the day while the mice were hungry) on days 1-5 (during habituation to the board and on training days 1 to 3 while mice were still adapting to the food reward). For habituation to the food reward, mice were given a capful (~1ml) each of food reward in their home

cage. Each day, 1 h after feeding for the day, mice were weighed and then injected with CBD or vehicle.

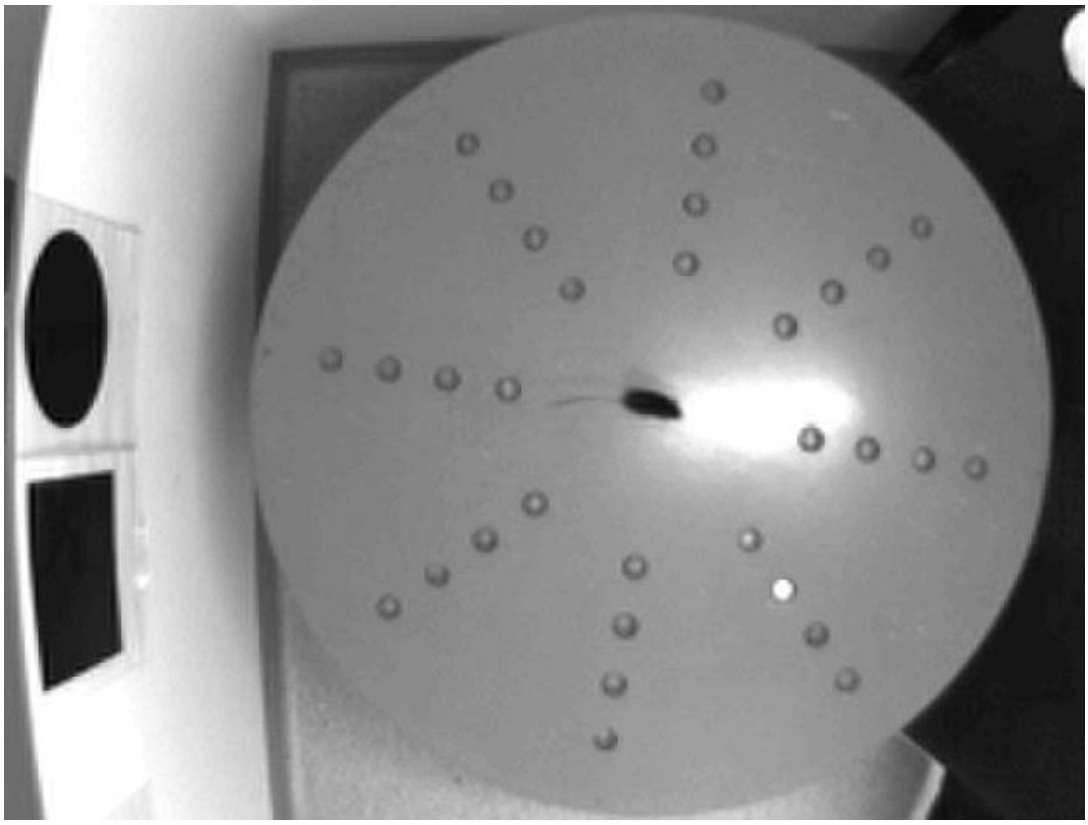

Figure 8: The cheeseboard paradigm; training trial.
A single well (lower right) was baited during training trials on the circular board. Two of the many spatial cues can be seen to the left of the apparatus.

Mice were habituated to the test room for 30 min each day and were tested in groups of 6-7. For each group of mice being tested, the filter top lids of the home cages were removed and placed under the corresponding cages prior to commencement of testing. The wire cage hopper was left on top of the home cage. This was implemented to minimise the disruption caused by adjusting the cage lids while retrieving the next mouse to be placed on the board. During all trials, mice were placed in the centre of the board using a start box, and once the box was removed mice were allowed to freely explore the board. All trials were video recorded with AnyMaze tracking software.

Mice were habituated to the blank side of the board for the first two days; mice were allowed to freely explore the board for 2 min for a total of three trials each day, with an ITI of 20 min.

The remainder of the test was performed on the side of the board containing the wells, each containing a bottle cap that was brushed with the food reward to remove possibility of odour cues distracting or guiding the mice. Days 3 to 7 consisted of three training trials per day (20 min ITI), during which spatial learning acquisition was made. During training trials, one cap (~1 ml) of food reward was available on the board, and the latency of the mice to find and drink the food reward was recorded with a stopwatch. Each trial was a maximum of 2 min; if the mouse hadn't found the food reward within these 2 min, it was gently guided to the well by the experimenter. All mice were allowed to drink from the baited well for no longer than 10 s. A total of five training days were run (i.e. total of 15 training trials). The position of the baited well was limited to the second and third row of wells (the inner and outer row of wells closest and furthest from the centre, respectively, were excluded to minimise the difference in distance that mice had to travel to the food reward; Figure 9). The baited well was the same for each day and each trial per mouse so that they would learn the location of the well, but a different well was baited between mice, and counterbalanced for genotype and treatment. The average latency of mice to find the reward for all three trials per day was analysed as a general indication of learning, while the first trial per day was analysed to assess long-term reference memory (retention of $\geq$ 24 h), and the average of trials 2 and 3 (each ~ 20 min following the previous trial) was analysed to assess intermediate-term memory (retention falling between that of short-term memory (2 min) and of long-term memory (24 h; Taglialatela et al. (2009)), i.e. ~20 min in this test, and spanning up to several hours). Mean speed was also assessed.

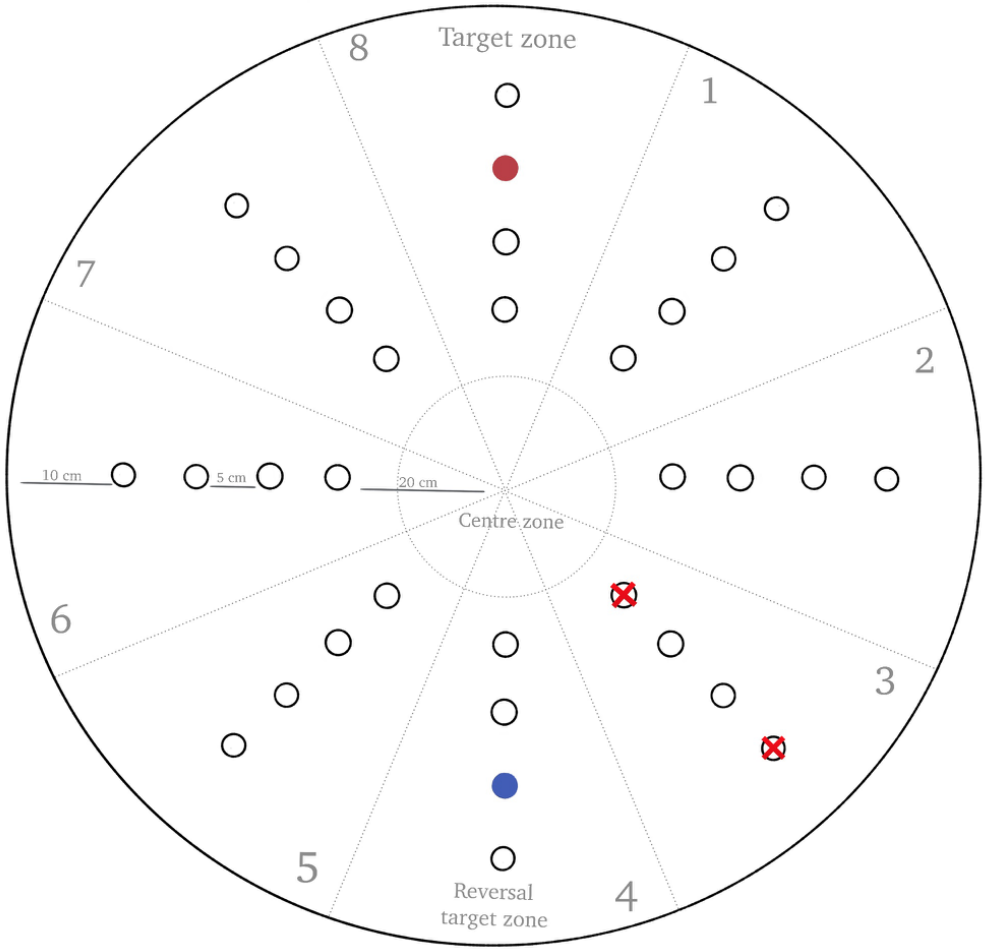

Figure 9: Cheeseboard schematic.
The cheeseboard has a circular centre zone and eight wedge shaped target zones, each containing four wells and extending to include the area proximal to the wells. One well is baited during training (coloured red in zone 8), but not during the probe trial, and the opposite well is baited during reversal training (coloured blue in zone 4). The inner and outer wells are never baited (indicated with a red cross in zone 3).

The CB probe trial for spatial memory was performed on day 8. For the probe, no food reward was placed on the board, but all caps were lightly brushed with the food reward. Mice were placed on the board and given 2 min to explore the board. The percentage of duration spent in the target zone for the full 2 min, and also for the first and second 30 s was calculated (to account for potential differences in behavioural flexibility rather than spatial memory; Grech et al. (2019)) post experiment using data from AnyMaze, and used as an indication of spatial memory. The target zone is

defined as the one zone out of the eight zones that during training held the baited well; the target zone therefore constituted 12.5% of the circular board and contained one row of four wells and the area adjacent to these wells (Figure 9). If mice spent significantly greater than 12.5% of their time in the target zone (greater than chance), then it was concluded that the mouse had developed a preference for this zone and therefore exhibited intact spatial memory. Increased time spent in the target zone in the first 30 s was indicative of intact retrieval memory, demonstrating that mice recall the position of the food reward from the day prior. Increased time in the target zone over next 30 s was indicative of perseverative behaviour, i.e. persistence to find the food reward in the target zone, while decreased time in the target zone over the second 30 s might be indicative of cognitive flexibility in adaptation to the lack of food reward (Grech et al. 2019). One *APPxPS1*-CBD mouse was excluded from probe analysis as it *froze* for 80 s (3x greater than any other mouse that showed some *freezing* behaviour).

A CB reversal (rCB) test was also completed; training began on day 9, 24 h post completion of the probe trial. For this, the opposite well on the board that was used in the initial training trials was baited (Figure 9). Mice were given four days of reversal training. Then on day 13, a reversal probe trial was carried out. The test procedures for initial training and probe trial and the reversal training and probe trial were identical. One *APPxPS1*-VEH and one *APPxPS1*-CBD mouse were included in the analysis of the whole 2 min of the test, but were excluded from analysis of the first and second 30 s of the reversal probe due to a technical issue that meant data for the whole 2 min was not able to be accurately split into 30 s bins for these two mice.

2.3.6 Prepulse inhibition (PPI)

There is recent evidence of reduced prepulse inhibition (PPI) in early stages of AD (Ueki et al. 2006), and in AD mouse models (Wang et al. 2012); thus this test was used to assess the acoustic startle response (ASR) and sensorimotor gating (also known as PPI). ASR is measured as the startle amplitude (arbitrary units) of an organism in response to an acoustic startling stimulus (in this test, a loud startle pulse of 100 or 120 decibels (dB)). The startle response can also be measured using visual or tactile stimuli, for example a flash of light, or puff of air to the eye (Swerdlow et al. 2001). Sensorimotor gating or PPI is a neurological mechanism that inhibits the processing of extraneous information, and in this test is the occurrence by which a weaker, non-startling prestimulus presented prior to a subsequent, stronger startling stimulus attenuates the startle response (Wang et al. 2012). In this test, the prestimulus was an acoustic prepulse of 74, 82 or 86 dB.

The apparatus consisted of a Plexiglas mouse enclosure (Figure 10) within a startle chamber (SR-Lab, San Diego Instruments, San Diego, USA), to which mice were habituated for 10 min twice per day (1 h ITI) over two consecutive days prior to the test day (total of four habituation trials of 10 min each on day 1 and 2). A consistent background noise of 70 dB was presented to mice in the enclosure for all habituation trials. On day 3, mice were returned to the apparatus for the PPI test. The test session was 35 min long and firstly consisted of a 5 min acclimation period to 70 dB background noise, followed by 97 trials presented in a pseudorandom order. The trials consisted of; 5 x 70 dB trials (no stimulus, background noise), 5 x 100 dB trials and 15 (3 blocks of 5 presented at the beginning, middle and end of the test) x 120 dB trials to test for ASR; and 72 trials of a prepulse (74, 82 or 86 dB) presented 32, 64, 128, or 256 ms (variable inter-stimulus interval; ISI) prior to a startle pulse of 120 dB to test

for PPI. Each prepulse x ISI combination was randomly presented six times, and the interval between each trial (ITI) varied randomly between 10 – 20 s. The startle responses to each trial were detected by the accelerometer as the average mean amplitude detected. ASR was calculated as the mean amplitude to all startle trials. ASR habituation was analysed by comparing the 5 blocks of 120 dB startle pulses at the beginning, the middle and the end of the PPI. Percentage PPI (%PPI) was calculated as [(mean startle response (120 dB) – PPI response)/mean startle response (120 dB)] x 100. %PPI was averaged across ISIs to produce a mean %PPI for each prepulse intensity.

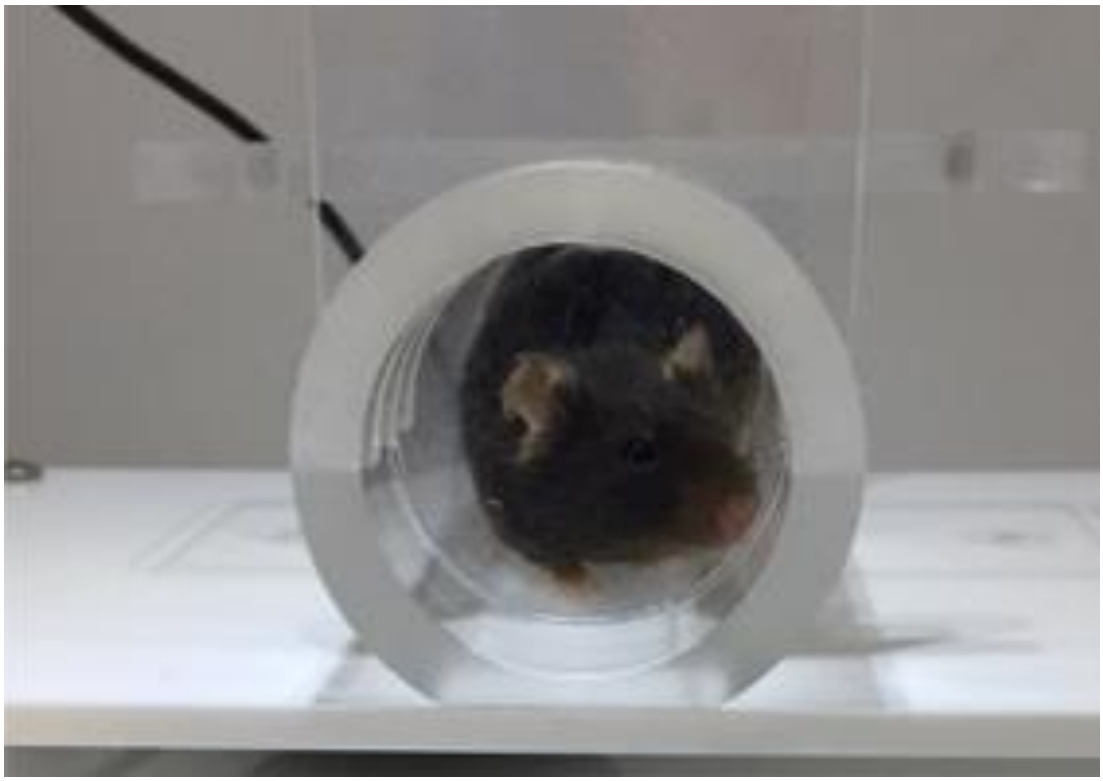

Figure 10: The prepulse inhibition mouse enclosure.
The reflective response of the mice to the startle pulse is recorded by an accelerometer attached underneath the enclosure.

2.4 Tissue collection

Following completion of behavioural testing, the test mice were euthanised and their brain tissue collected for future molecular analysis (for time reasons this was not possible to be carried out as part of this *Master of Research* Project). Two key pathological signs of AD are the presence of extracellular deposits of Aβ and intracellular NFT of hyperphosphorylated tau in brains of both human patients and *APPxPS1* transgenic mice (Aso, Andrés-Benito & Ferrer 2016). Thus, brain tissue was collected for future analysis of these proteins to determine the effect of CBD on AD-relevant brain pathology in this mouse model, to accompany the already completed behavioural analysis. It is expected that future molecular analysis will involve the quantification of AD-relevant proteins including soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ through an enzyme-linked immunosorbent assay (ELISA) in the PFC, hippocampus and cerebellum of mice as published previously (Cheng et al. 2014c). Future analysis could also investigate levels of ionized calcium binding adaptor molecule 1 (IBA-1) to determine microglial activity. We could also investigate the cytokines TNF-α and interleukin 1 beta (IL-1β) to determine levels of neuroinflammation.

2.4.1 Reagent preparation

2L of 1x phosphate buffered saline (PBS) was prepared by dissolving 16 g sodium chloride (NaCl), 0.4 g potassium chloride (KCl), 2.88 g sodium phosphate dibasic (Na_2HPO_4) and 0.48 g potassium phosphate monobasic (KH_2PO_4) in water, which was then refrigerated at 4 °C. The final concentration of 1x PBS was 137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4 and 1.8 mM. KH_2PO_4.

200 mL 4% (w/v) paraformaldehyde (PFA) was prepared in a ventilated fume hood by adding 8 g powdered PFA to 200 mL 1x PBS that had been heated to 60 °C. The PFA solution was maintained at 60 °C on a hotplate and mixed using a magnetic stirrer

for approximately 1 h or until clear. Once dissolved, PFA was filtered through filter paper and stored at 4 °C in a spark proof fridge.

100 mL of 30% (w/v) sucrose was made by dissolving 30 g white sugar in 100 mL PBS and was stored at 4 °C. Fresh reagents were made for each cohort of mice one day prior to sacrifice. All chemicals used in the preparation of reagents are from Sigma-Aldrich Co., St Louis, USA.

2.4.2 Anaesthesia and euthanasia

Mice were individually anaesthetised in an anaesthesia chamber containing isoflurane (Veterinary Companies of Australia, Sydney, Australia) soaked cotton wool placed underneath a metal plate that separated the paws of the mice from the liquid anaesthetic. Lack of reflexes including the eye blink, toe pinch and tail pinch determined adequate anaesthesia. Anesthetised mice were then transcardially perfused by an experienced experimenter with 1x PBS to remove blood and collect tissue and then decapitated for euthanasia.

Briefly, the anaesthetised mouse was laid out flat on its back on a dissecting board. The snout of the mouse was placed into a nose cone containing a cotton ball soaked in isoflurane to maintain anaesthesia, and the limbs were secured with tape. The heart was exposed using cuts along the sides of the torso, the abdomen and then the thorax of the animal, with the diaphragm cut within 20 s of the procedure. A 23G x 3/4" butterfly needle was inserted into the left ventricle to puncture the heart. The butterfly needle was connected to a tube that connected to the perfusion pump, and which had previously been flushed with 1x PBS to ensure that there were no trapped air bubbles. Next, the right atrium was cut, and the perfusion pump activated at a flow rate of a maximum of 10 ml/min. The circulatory system was flushed until the exit fluid was

clear and the liver, kidneys and other organs were blanched (within 2 min). The mouse was then decapitated using scissors.

2.4.3 Sample collection

Tail tips were collected from the euthanised mice and snap frozen in liquid nitrogen and stored at -80 °C for future genotype confirmation analysis, which has not yet been performed. Brains were quickly removed and rinsed in ice-cold PBS before being sagittally divided on ice. The left hemisphere was dissected, with the olfactory bulb, cerebellum, PFC and hippocampal samples being removed and snap frozen in liquid nitrogen before being moved to -80 °C for long-term storage and future molecular analysis. The right hemisphere was fixed in 4% PFA and refrigerated at 4 °C for 24 h, before being washed in 1x PBS and then placed into a 5ml tube of 30% sucrose. Once the right hemisphere had sunk to the bottom of the tube (approximately 24 h), indicating that brains had absorbed the sucrose to signify adequate cryoprotection, it was removed from the sucrose and slowly frozen (to prevent cracking); the hemisphere was patted dry of sucrose, and held over liquid nitrogen in a plastic cap until frozen, before being stored at −80 °C for future analysis.

2.5 Statistical analysis

Prior to analysis of behavioural parameters, three-way analysis of variance (ANOVA) was used to ensure no ‘cohort’ effects on ‘genotype’ or ‘treatment’ for the main parameter of each test. As no ‘cohort’ effects were found (data not shown) all further analysis considered the three cohorts as one. Analysis of measured parameters was performed using two-way ANOVA to determine main effects of between-subject factors ‘genotype’, ‘treatment’ and ‘genotype’ by ‘treatment’ interactions. Three-way repeated measures (RM) ANOVA was also used to investigate repeated measures

effects of within-subject factors of ‘time’ (LD), ‘object’ (NORT), ‘time’ (CB), and ‘startle pulse intensity,’ ‘startle block’ and ‘prepulse intensity’ (all PPI). One sample t-tests were also used for LD, NORT and CB probe to determine whether a specific behaviour was above chance levels (i.e. 50% for LD and NORT – 12.5% for CB). Significant differences were determined when $p < .05$. F-values and degrees of freedom are presented for ANOVA and significant effects of ‘genotype’ are shown in figures and tables by ‘*’ ($*p < .05$, $**p < .01$, and $***p < .001$), and significant effects of ‘treatment’ are shown by ‘#’ ($\#p < .05$). Significant RM results are indicated by ‘^’ ($\wedge p < .05$, $\wedge\wedge p < .01$, and $\wedge\wedge\wedge p < .001$). Significant t-test results are also shown by ‘+’ ($+p < .05$, $++p < .01$, and $+++p < .001$). Trends were reported when $.05 < p < .09$, and all other non-significant data were reported as $p > .05$. Data are shown as means ± standard error of means (SEM). All statistical analyses were conducted using SPSS 25.0 for Mac.

Chapter 3: Results

3.1 Locomotion and exploration

APPxPS1 mice displayed increased total distance travelled in the LD test [two-way ANOVA for 'genotype': $F(1,46) = 9.2$, $p = .004$] and this increase in locomotion was not affected by treatment (i.e. no 'genotype' by 'treatment' interaction, $p > .05$; Table 2 and Figure 11A). Importantly, this hyper-locomotive phenotype of *APPxPS1* females was replicated when analysing distance travelled in the dark zone, which is the locomotion measure of the LD paradigm least affected by anxiety [$F(1,46) = 16.3$, $p < .001$; Table 2 and Figure 11B]. All mice habituated to the LD arena across time as indicated by reduced locomotion across 5-min bins in the whole LD arena [RM ANOVA for 'time': $F(1,46) = 117.8$, $p < .001$; Figure 11A] as well as the dark zone only ['time': $F(1,46) = 34.5$, $p < .001$; Figure 11B]. Neither genotype nor CBD treatment impacted on the habituation of the locomotor response (no interaction effects of 'genotype' or 'treatment' with 'time': all p's > .05).

Table 2: Locomotion in the light dark test.
Data shown for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM. Main 'genotype' effects across treatment conditions are presented as '*' (**$p < .01$ and ***$p < .001$).

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Total distance [m]**	32.5 ± 1.6	40.0 ± 2.9	32.4 ± 1.5	38.7 ± 3.1
Dark zone distance [m]***	17.0 ± 1.1	21.4 ± 1.8	17.1 ± .9	23.4 ± 1.5

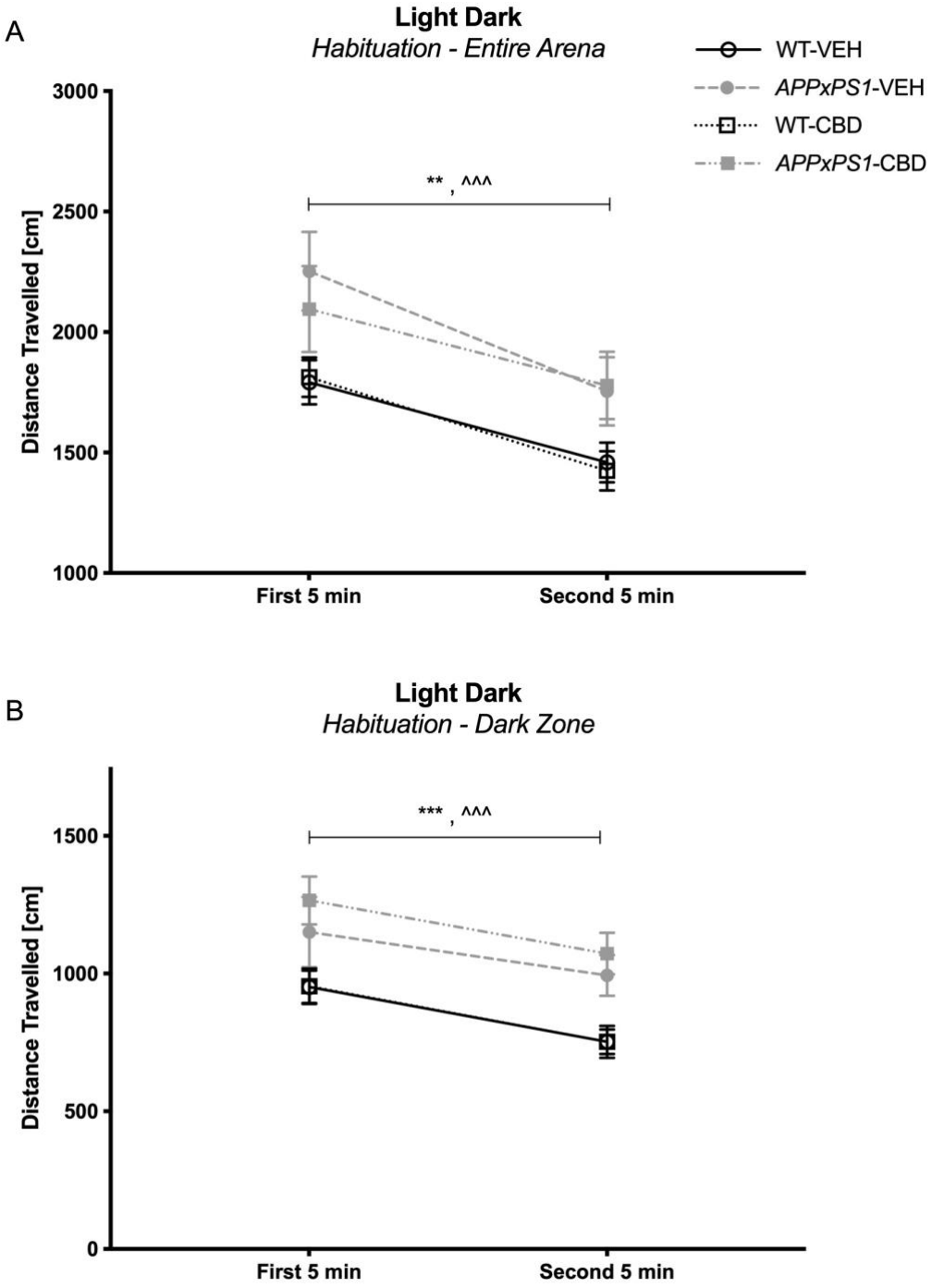

Figure 11A-B: Habituation in the light dark test.
A) Habituation to the entire apparatus and **B)** to the dark zone, as distance travelled [m] over time. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant main effects of 'genotype' are indicated by '*' (**$p < .01$ and ***$p < .001$), and repeated measures effects of 'time' by '^' (^^^$p < .001$).

Exploration was determined by the number of *rearing* events in the LD arena. Interestingly, mice treated with CBD exhibited an increased frequency of exploratory *rearing* in the dark zone of the LD compared to vehicle-treated mice [two-way ANOVA for 'treatment': $F(1,46) = 5.5$, $p = .02$; Figure 12], and this was not affected by genotype (no 'genotype' by 'treatment' interaction and no main effect of 'genotype', all *p*'s > .05), This CBD-induced increase in *rearing* activity was absent in the light zone and the full LD arena and was also not affected by 'genotype' (all *p's* > .05 for; data not shown).

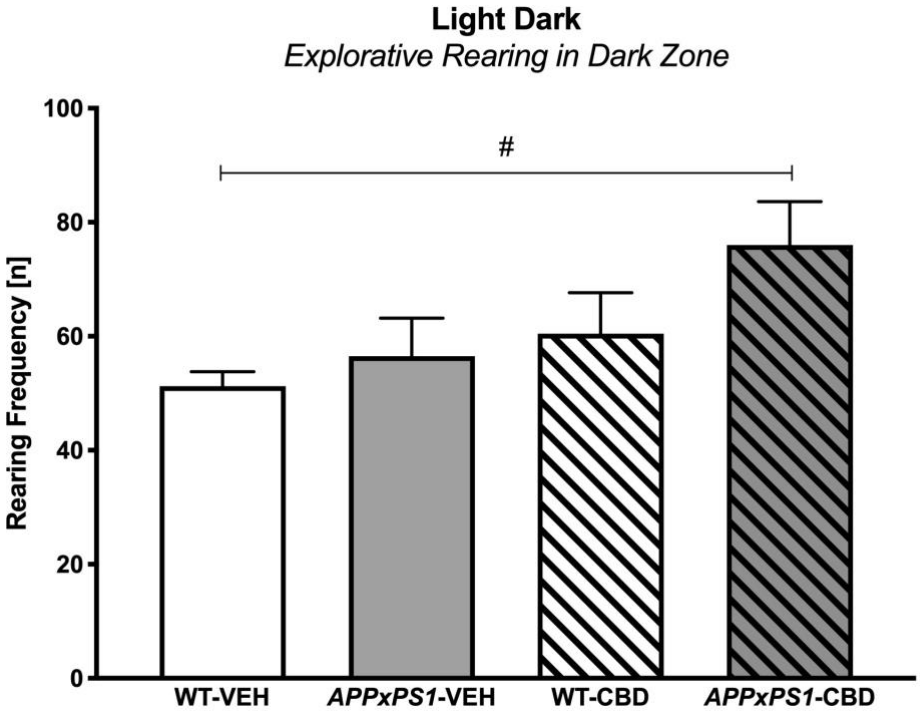

Figure 12: Exploration in the light dark test.
Rearing frequency [n] in the dark zone. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant main effects of 'treatment' are indicated by '#' (#*p* < .05).

3.2 Anxiety

Two-way ANOVA indicated that there was an overall effect of 'genotype' for the anxiety-related parameters of the LD test. In particular, *APPxPS1* mice spent a significantly higher percentage of the test time in the traditionally less aversive dark zone compared to WT females [$F(1,46) = 4.9$, $p = .03$; Figure 13A] and they also tended to travel more in the dark zone than their control littermates [i.e. percentage distance travelled in the dark zone: $F(1,46) = 3.6$, $p = .06$; Figure 13B]. However, a comprehensive analysis of the anxiety parameters in the LD test revealed that WT mice did not show the expected preference for the dark zone. Indeed, one sample t-test for the percentage time spent and percentage distance travelled in the dark zone indicated that neither WT-VEH, *APPxPS1*-VEH nor WT-CBD mice demonstrated a significant preference for the dark zone above chance levels (50%), i.e. these experimental groups spent an equal amount of time (and travelled equally far) in both zones of the LD arena [percentage time in dark zone: WT-VEH; $t(14) = 1.1$, $p = .3$, *APPxPS1*-VEH; $t(9) = 1.0$, $p = .3$, WT-CBD; $t(12) = 1.7$, $p = .1$ - percentage distance travelled in dark zone: WT-VEH; $t(14) = -.6$, $p = .5$, *APPxPS1*-VEH; $t(9) = .2$, $p = .8$, WT-CBD; $t(12) = -1.3$, $p = .2$; Figure13A-B]. Only *APPxPS1* mice chronically treated with CBD exhibited a preference for the dark zone [percentage time: $t(11) = 3.0$, $p = .01$ - percentage distance travelled; $t(11) = 4.9$, $p < .001$; Figure13A-B].

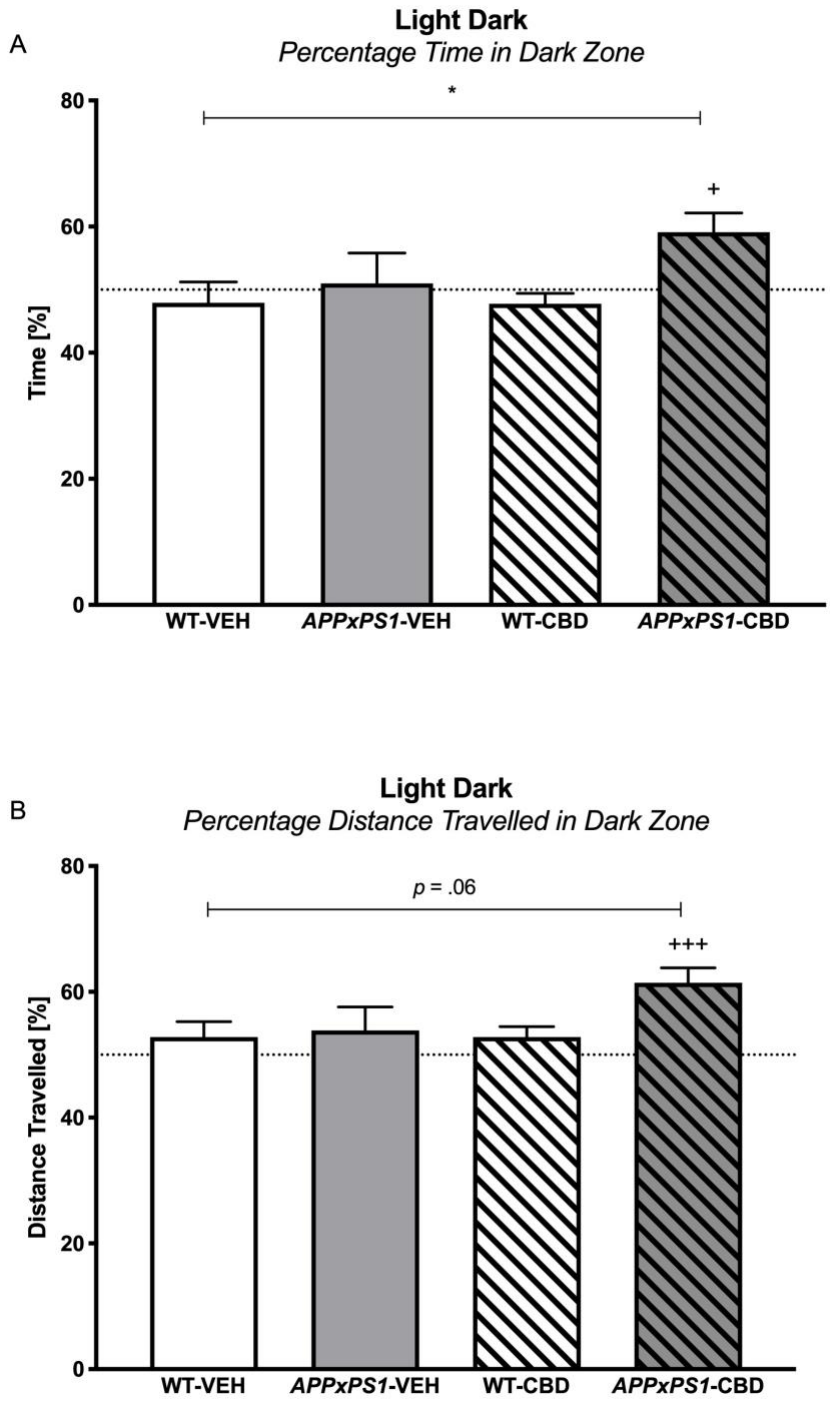

Figure 13A-B: Anxiety measures in the light dark test.
A) Percentage of time spent in the dark zone [%] and **B)** percentage of total distance travelled in the dark zone [%]. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant two-way ANOVA 'genotype' effects are indicated with '*' (*p < .05), and trend for 'genotype' effects indicated by 'p = .06'. Single sample t-test results against chance levels are indicated by '+' (+p < .05, +++p < .001).

As WT mice did not show a preference for the dark zone using the automatic tracking system and zone settings provided by the manufacturer (shown in Figure 14A), we also tested LD zone preferences when considering an exclusion zone around the dark zone entry area (Figure 14B) to avoid that potential risk-assessment behaviours (i.e. *stretch-attend postures*) had confounded the LD zone preference scores. Based on this central exclusion zone, behaviours of mice in the "new dark zone" and "new light zone" were used to determine zone preferences. However, adding an exclusion zone did not change the original finding, i.e. most experimental groups did not show a preference for the dark zone (data not shown); that is, only the *APPxPS1*-CBD experimental group showed a preference for the dark zone using these new settings.

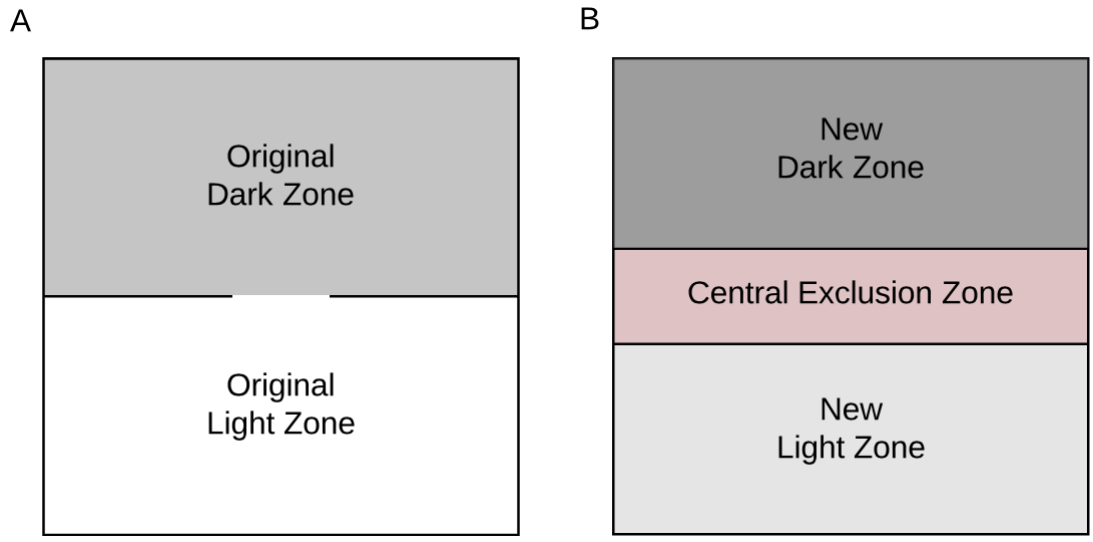

Figure 14A-B: Light dark test zone settings.
A) The original MED Associates Inc. zone settings used in the analysis of anxiety (*dark zone location; X:0 and Y:17, and light zone location; X:0, Y:8.5. Size of each dark and light zone; 17 x 8.5 beams, 26.99 x 13.49 cm*) and **B)** the new zone settings used for the reanalysis of the light dark (LD) test (*dark zone location; X:0 and Y:17, central exclusion zone location; X:0, Y:9.5, and light zone location; X:0, Y:7.5. Size of each dark and light zone; 17 x 7.5 beams, 26.99 x 11.91 cm. Size of central exclusion zone; 26.99 x 3.18 cm*).

3.3 Motor function

The motor abilities of mice in the pole test were not affected by the *APPxPS1* transgenes nor by CBD treatment, i.e. no significant main effects were found for the measures inversion time, latency to reach the platform, and the time to descend once inverted [all *p's* > .05; Table 3]. Similarly, in the accelerod, two-way ANOVA found no effects of 'genotype' or 'treatment' for the average latency to fall from the accelerod [all *p*'s > .05; Figure 15A]. However, a significant effect of 'genotype' was found when comparing the worst performance of test mice across trials (i.e. the shortest latency across the four trials); *APPxPS1* mice fell from the accelerod significantly earlier than WT mice [$F(1,46) = 7.1$, $p = .01$; Figure 15B]. This effect of genotype was not affected by CBD (no 'genotype' by 'treatment' interaction, $p > .05$). There was no genotype difference in the latency of mice to fall from the accelerod when considering the best performance (i.e. the longest latency across four trials: $p > .05$; Figure 15C). CBD treatment had no effect on any accelerod performance measures (all *p's* > .05).

Table 3: Motor functions in the pole test.
Data shown for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM.

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Inversion time [s]	13.8 ± 2.9	14.0 ± 3.8	7.6 ± 1.2	10.7 ± 2.5
Latency to platform [s]	31.6 ± 4.4	32.2 ± 5.2	22.7 ± 1.8	28.7 ± 3.9
Time to descend once inverted [s]	17.8 ± 2.0	18.2 ± 2.7	15.0 ± 1.3	18.0 ± 2.2

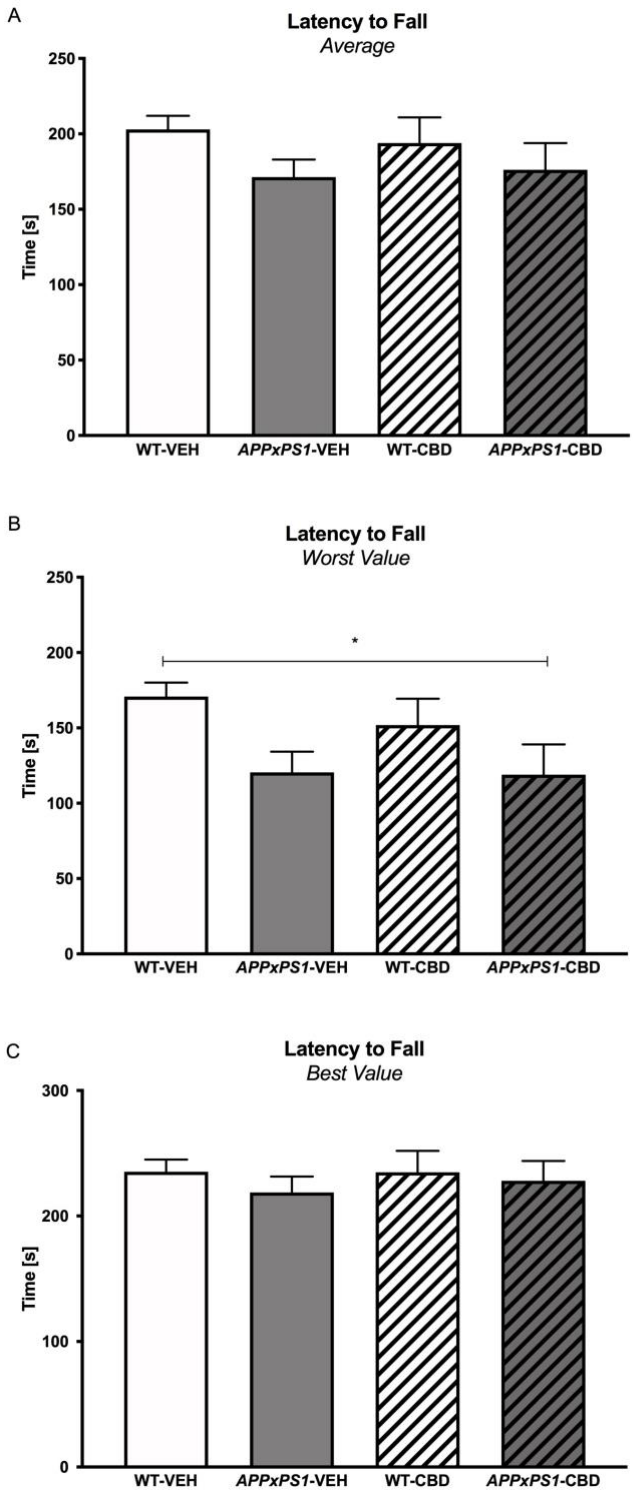

Figure 15A-C: Motor performance in the accelerod.
A) The average latency [s], **B)** worst performing latency [s] and **C)** best performing latency [s] to fall from the accelerod of the four trials. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant 'genotype' effects are indicated with '*' (*$p < .05$).

3.4 Cognition

3.4.1 Object recognition memory (NORT)

In the NORT testing trial, all experimental groups, excluding *APPxPS1*-VEH mice, had a preference for the novel object which was significantly above chance level (50%), as indicated by one sample t-tests for the time spent *nosing* the novel object (as a percentage of the total time spent *nosing* both the novel and familiar objects) [WT-VEH; $t(13) = 4.5$, $p = .001$, *APPxPS1*-VEH; $t(9) = .5$, $p = .6$, WT-CBD; $t(12) = 2.8$, $p = .02$, *APPxPS1*-CBD; $t(9) = 2.6$, $p = .03$; Figure 16].

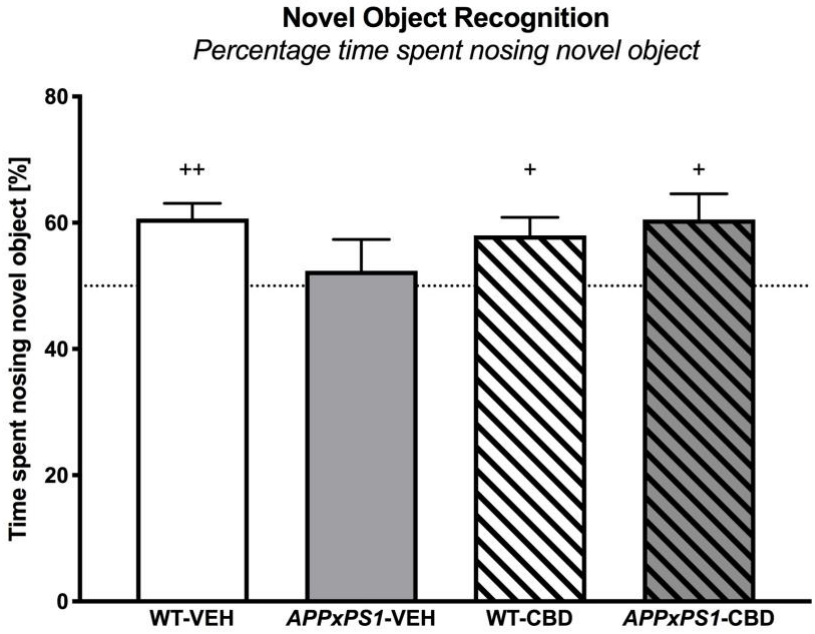

Figure 16: Novel object recognition.
The percentage of time spent *nosing* the novel object in the NORT. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant t-test results are indicated with '*' (*$p < .05$, **$p < .01$).

3.4.2 Spatial memory test – task acquisition and mean speed

In the CB training trials, all mice demonstrated successful task acquisition as they learned the position of the baited well. This was indicated by a reduced latency to find the food reward over time, when averaged across the three trials per day [three-way RM ANOVA for 'time': $F(4,180) = 88.6$, $p < .001$; Figure 17A], and also when considering intermediate-term memory (average of trials 2 and 3: $F(4,180) = 59.3$, $p < .001$; Figure 17B). Although *APPxPS1* mice took longer per day to find the reward (main effect of 'genotype' for average of all 3 trials: $F(1,45) = 6.8$, $p = .01$ - for average of trial 2 and 3: $F(1,45) = 11.1$, $p = .002$; Figure 17A-B), their decreasing latency to find the food reward across trials and days paralleled that of WT mice, indicating learning was similar across genotypes (no interaction of 'time' with 'genotype', $p > .05$). When looking at the training performance of the mice based on reference memory only (i.e. comparing trial 1 of each day only), all mice exhibited a decrease in latency to find the food reward over time ['time': $F(4,180) = 41.6$, $p < .001$; Figure 17C] and this was not affected by genotype or CBD treatment (no interactions with 'time': all *p's* > .05). To analyse the *APPxPS1* genotype effect on training latencies further, analysis of the mean speed of mice across the CB training trials was carried out. Indeed, *APPxPS1* transgenic mice were slower on the board than their WT littermates, regardless of treatment [three-way RM ANOVA for 'genotype': $F(1,45) = 24.5$, $p < .001$; Figure 18A]. There were no effects of 'treatment' and no interactions between 'genotype' and 'treatment' on the speed during the training trials (all *p's* > .05). Interestingly, the mean speed of all mice during CB training increased over days [three-way RM ANOVA for 'time': $F(4,180) = 7.8$, $p < .001$; Figure 18A], with no interaction of 'genotype' or 'treatment' between 'time' (*p*'s >.05).

During reversal training, all mice adapted to the alteration in the food reward position and showed decreasing latencies to find the food reward over days [three-way RM ANOVA for 'time' across three trials: $F(3,135) = 47.2$, $p < .001$ - across trials 2 and 3: $F(3,135) = 20.5$, $p < .001$ - across trial 1 only: $F(3,135) = 38.9$, $p < .001$]. There was no 'time' by 'genotype' or 'time' by 'treatment' interactions for any of these learning performances (all *p's* > .05; Figure 19A-C]. A main effect of 'genotype' [across three trials: $F(1,45) = 17.9$, $p < .001$ - across trials 2 and 3: $F(1,45) = 22.3$, $p < .001$ - across trial 1 only: $F(1,45) = 7.0$, $p = .007$] indicated that *APPxPS1* mice took generally longer to find the reward than WT mice. Similar to the initial training, when analysing the mean speed of mice during the rCB training trials, *APPxPS1* mice were slower on the board than their WT littermates, regardless of treatment [main effect of 'genotype': $F(1,45) = 17.2$, $p < .001$; Figure 18B]. There were no effects of 'treatment' and no interactions between 'genotype' and 'treatment' for the speed during the rCB training trials (all *p's* > .05). Different to the initial training period, the mean speed of all mice during rCB training did not increase ($p > .05$), with the mean speed during rCB remaining constant across days.

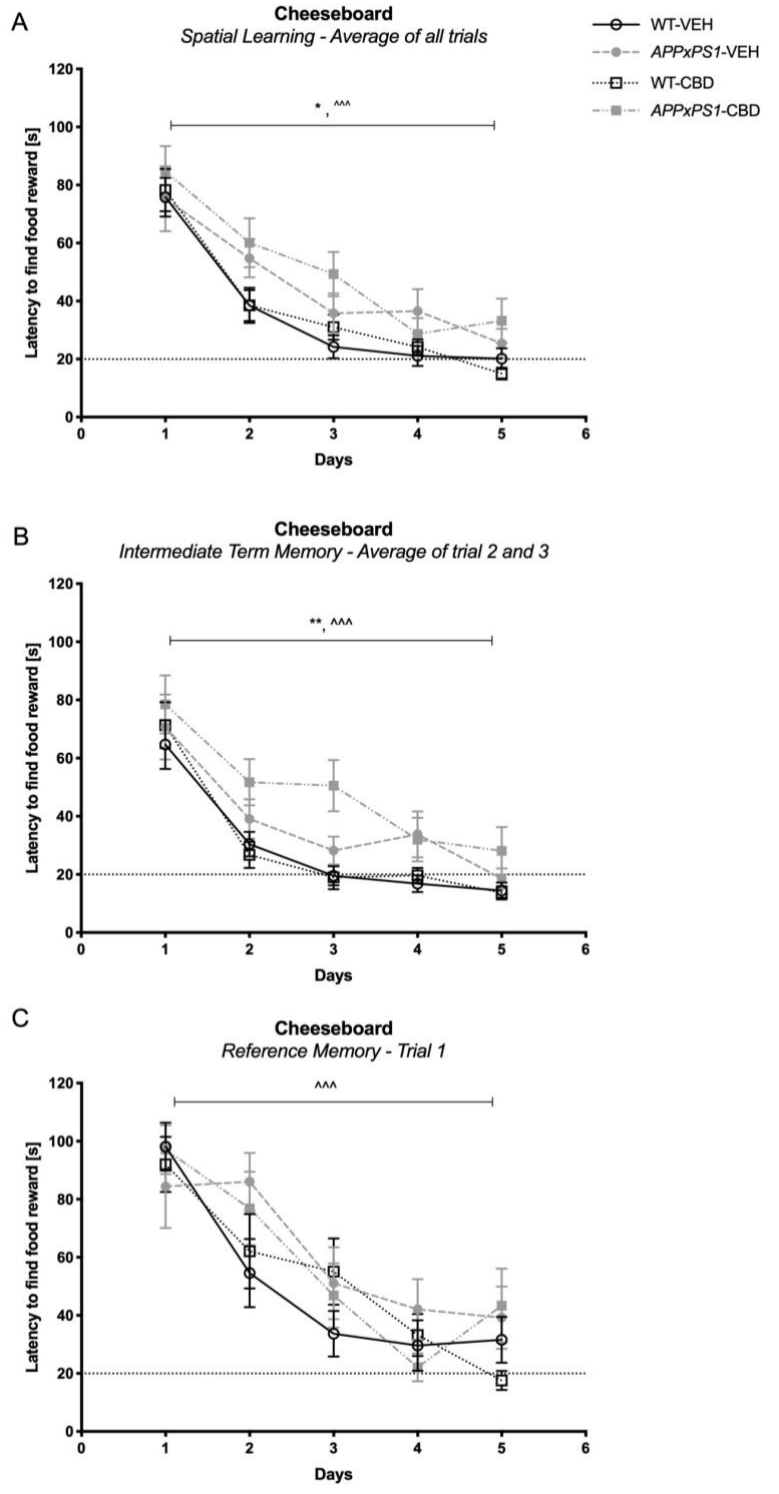

Figure 17A-C: Spatial learning in the cheeseboard (CB).
Latency [s] to find the food reward during CB training **A)** averaged across all three trials, **B)** averaged across trials 2 and trial 3, and **C)** across trial 1. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant 'genotype' effects are indicated by'*' (*$p < .05$, and **$p < .01$) and learning (effect of RM 'time') is indicated by '^' (^^^$p < .001$).

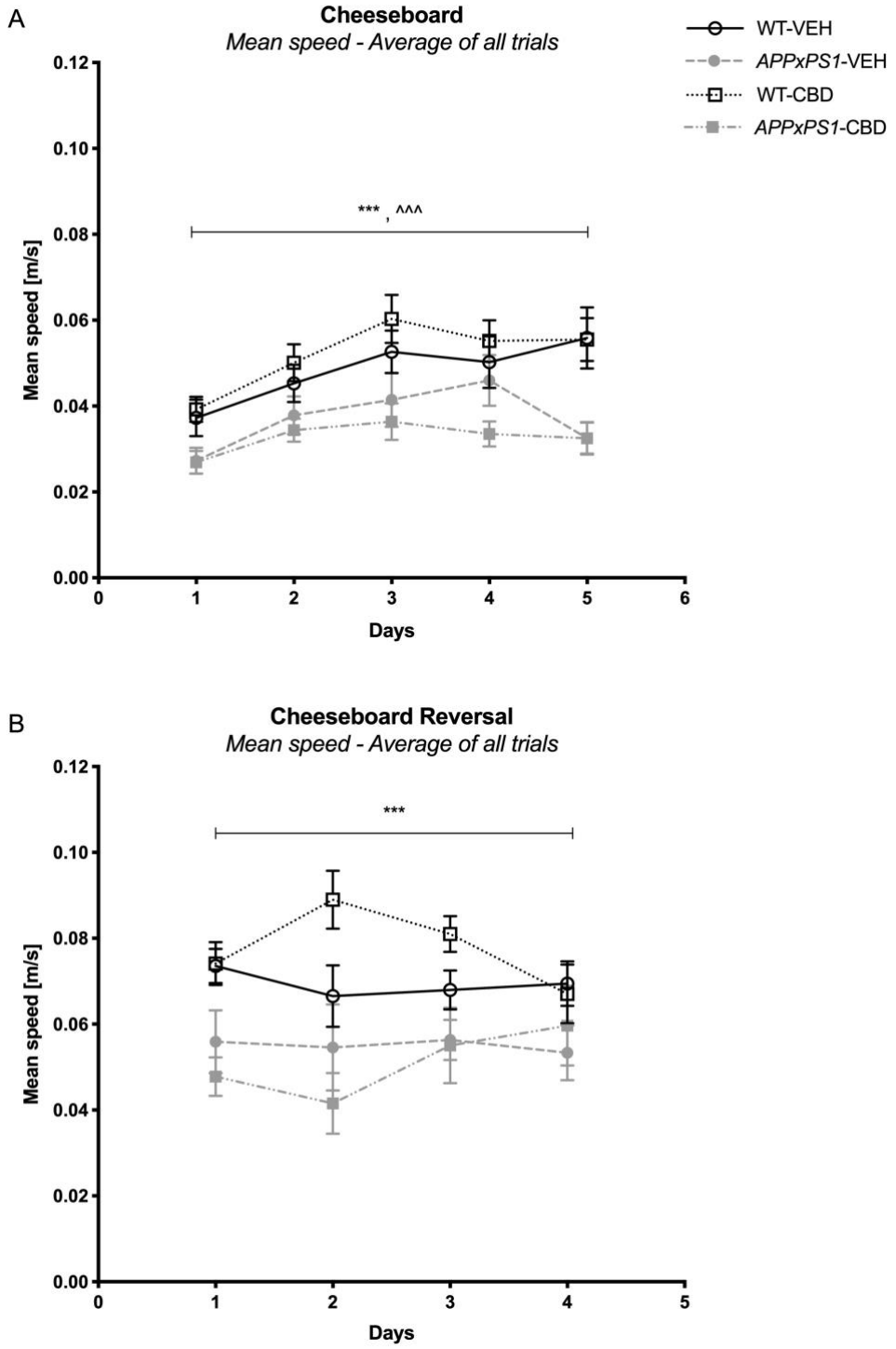

Figure 18A-B: Mean speed during cheeseboard (CB) and reversal cheeseboard (rCB) training.

Mean speed [m/s] averaged across all three trials for **A)** CB training and **B)** rCB training. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant 'genotype' effects are indicated by '*' (***$p < .001$) and effect of RM 'time' is indicated by '^' (^^^$p < .001$).

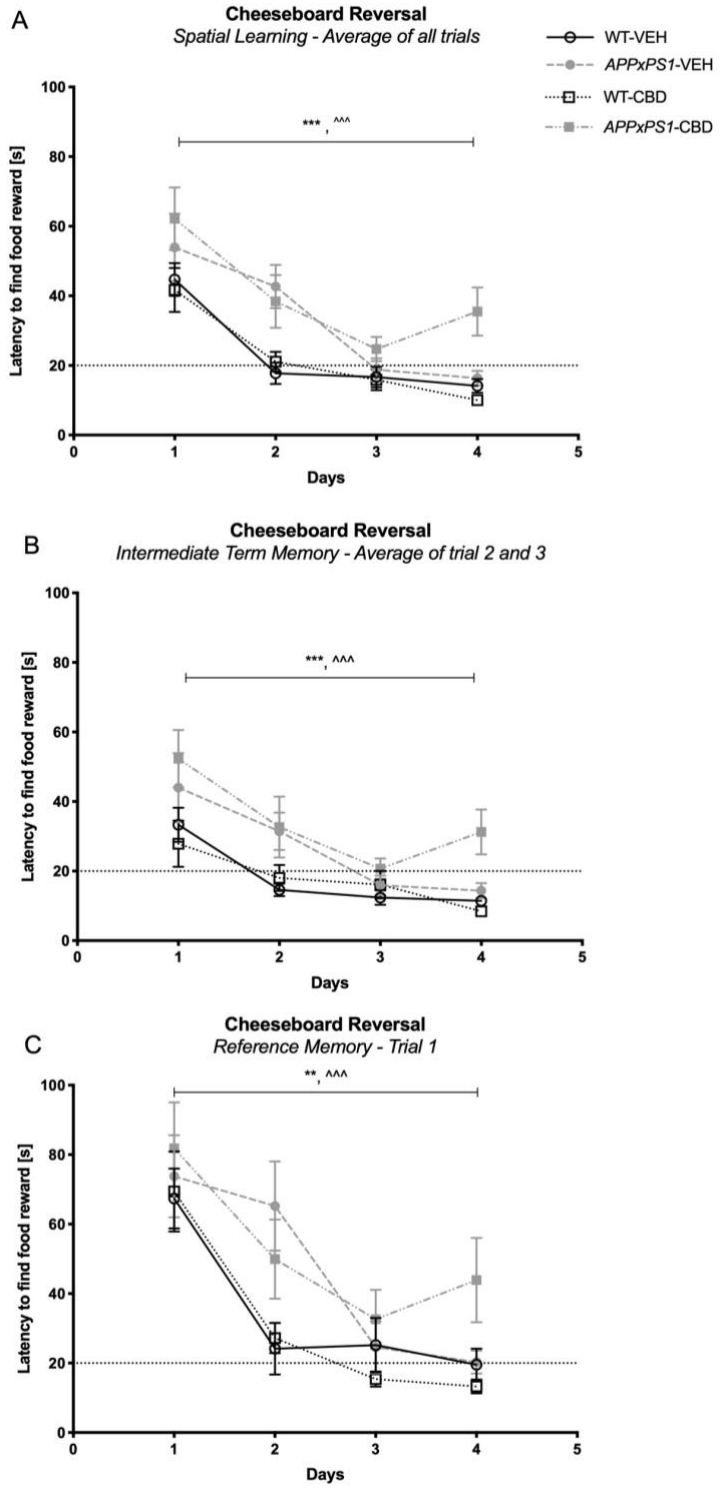

Figure 19A-C: Spatial learning in the reversal cheeseboard (rCB).
Latency [s] to find the food reward during rCB training **A)** averaged across all three trials, **B)** averaged across trials 2 and trial 3, and **C)** across trial 1. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant 'genotype' effects are indicated by '*' (**$p < .01$, and ***$p < .001$) and learning (effect of RM 'time') is indicated by '^' (^^^$p < .001$).

3.4.3 Spatial memory, memory retrieval and perseverance

During the CB probe trial, all mice spent greater than 12.5% of the total 2 min test time in the target zone, demonstrating that all mice regardless of 'genotype' or 'treatment' successfully recalled the position of the baited well as they showed a preference for the target zone greater than chance [one sample t-test: WT-VEH; $t(14) = 4.4$, $p = .001$, *APPxPS1*-VEH; $t(9) = 4.2$, $p = .002$, WT-CBD; $t(12) = 5.5$, $p < .001$, *APPxPS1*-CBD; $t(9) = 3.4$, $p = .007$; Figure 20A]. It was observed that some mice did not leave the centre zone immediately, and therefore did not spend the entire 2 min of the probe trial exploring the board. Therefore, a secondary calculation was carried out to ensure that the data presented was representative of the actual test time that mice spent exploring and was not skewed by an extended latency to leave the central start zone. This was calculated as [time (s) in target zone/(120 s – latency (s) to leave the centre zone] x 100. Importantly, this secondary measure did not change the significance findings of the initial target zone results (data not shown).

The first 30 s and second 30 s bins of the CB probe were also analysed to investigate retrieval memory and perseverance, respectively. One sample t-test for the time spent in the target zone as a percentage of 30 s indicates that all mice demonstrated intact retrieval memory in the first 30 s [WT-VEH; $t(14) = 4.8$, $p < .001$, *APPxPS1*-VEH; $t(9) = 3.1$, $p = .01$, WT-CBD; $t(12) = 5.3$, $p < .001$, *APPxPS1*-CBD; $t(9) = 3.3$, $p = .009$; Table 4]. Interestingly, when investigating the second 30 s time bin as an indication of perseverance, it was evident that while WT mice persevered to find the food reward, i.e. spent significantly more than 12.5% of the second 30 s in the target zone [one sample t-test: WT-VEH; $t(14) = 3.3$, $p = .005$, WT-CBD; $t(12) = 3.3$, $p = .006$], *APPxPS1* mice regardless of treatment did not [*APPxPS1*-VEH; $t(9) = 1.8$, $p = .1$, *APPxPS1*-CBD; $t(9) = 1.2$, $p = .3$; Table 4].

Similar analyses were also conducted for the rCB probe. All experimental groups (weak trend for *APPxPS1*-CBD mice), had a preference for the target zone [WT-VEH; t(14) = 2.9, p = .01, *APPxPS1*-VEH; t(9) = 2.5, p = .03, WT-CBD; t(12) = 4.6, p = .001, *APPxPS1*-CBD; t(10) = 1.9, p = .09; Figure 20B]. No change in results became apparent when taking into consideration the latency of mice to leave the central start zone (data not shown). In addition, all mice demonstrated intact retrieval memory in the reversal probe (first 30 s block) [WT-VEH; t(14) = 2.8, p < .01, *APPxPS1*-VEH; t(8) = 3.4, p = .01, WT-CBD; t(12) = 4.3, p = .001, *APPxPS1*-CBD; t(9) = 2.7, p = .03; Table 4]. However, as seen in the CB probe, while WT mice persevered to find the food reward in the second 30 s block [WT-VEH; t(14) = 3.7, p = .002, WT-CBD; t(12) = 2.8, p = .02], *APPxPS1* mice again did not [*APPxPS1*-VEH; t(8) = 1.8, p = .1, *APPxPS1*-CBD; t(9) = 1.7, p = .1; Table 4].

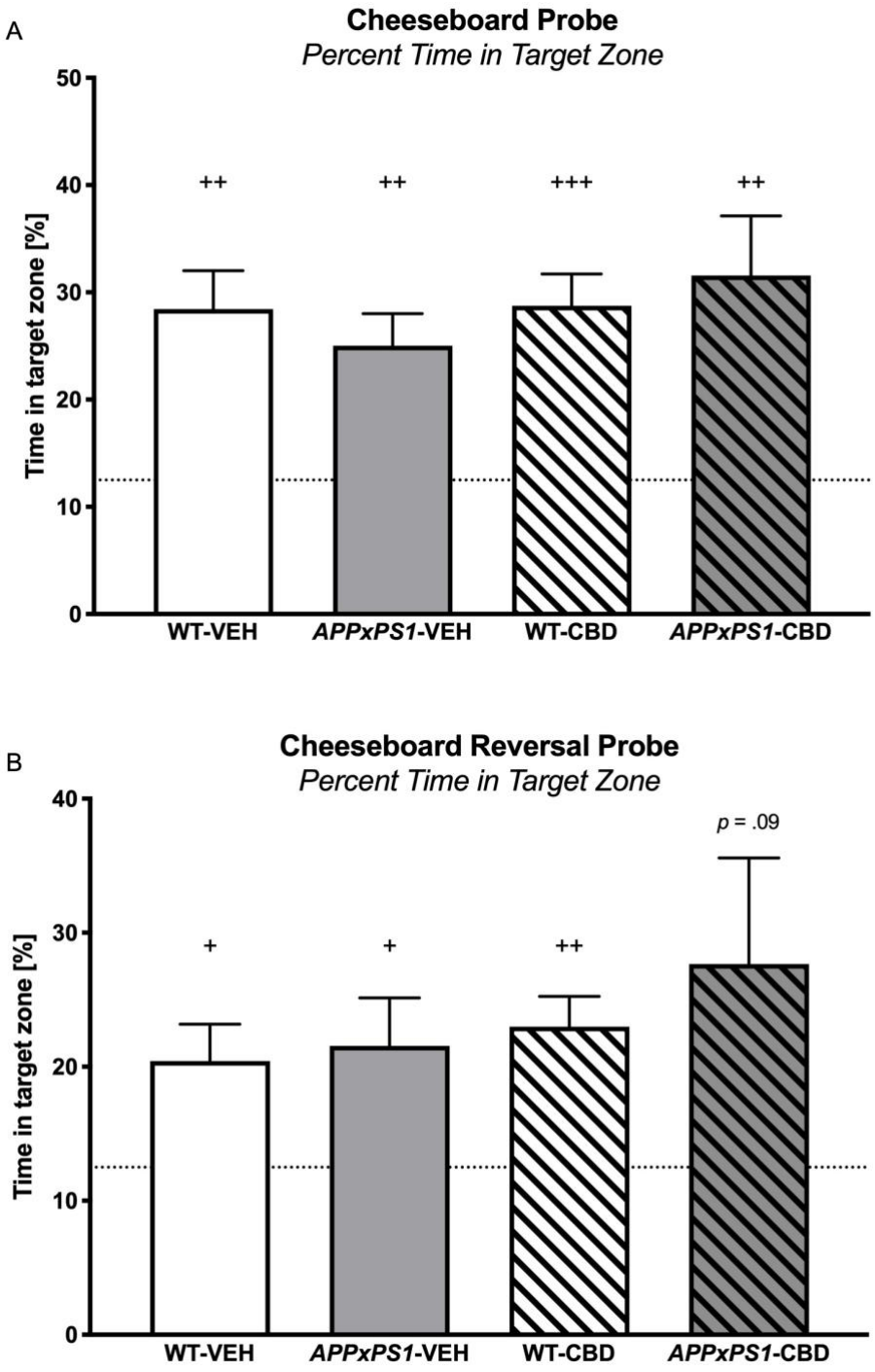

Figure 20A-B: Spatial memory in the cheeseboard (CB) probe and reversal cheeseboard (rCB) probe.
A) Percentage of time spent [%] in the target zone for the CB probe and **B)** for the rCB probe. Data for non-transgenic wild type-like (WT) control and double transgenic *APPSwe/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant t-test results are indicated by '+' (+$p < .05$, ++ $p < .01$ and +++$p < .001$) and a trend is indicated by '$p = .09$'

Table 4: Retrieval memory and perseverance in the cheeseboard (CB) probe and reversal cheeseboard (rCB) probe.
Data shown as percentage of time spent in the target zone in the first and second 30 s of each probe test for non-transgenic wild type-like (WT) control and double transgenic APPSwe/PS1ΔE9 (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM. Significant t-test results are shown by '+' (+$p < .05$, ++$p < .01$, and +++$p < .001$). First 30 s block data represents retrieval memory, second 30 s block data represents perseverance.

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
CB probe - % time spent in target zone				
First 30 s	27.5 ± 3.1+++	30.3 ± 5.8+	34.8 ± 4.2+++	34.2 ± 6.5++
Second 30 s	31.8 ± 5.9++	28.9 ± 8.9	22.6 ± 3.0++	21.0 ± 7.1
rCB probe - % time spent in target zone				
First 30 s	29.5 ± 6.0+	27.1 ± 4.3+	30.2 ± 4.1++	34.0 ± 8.0+
Second 30 s	26.3 ± 3.8+	19.1 ± 3.8	21.6 ± 3.2+	27.2 ± 8.7

3.5 Prepulse inhibition

3.5.1 Acoustic startle response

The ASR of all mice was not significantly different as there were no main effects of 'genotype' or 'treatment' (all *p*'s > .05). All mice responded to the startle pulses with increasing startle intensities (70 dB, to 100 dB, to 120 dB), with the 120 dB startle pulse producing the greatest startle responses [RM ANOVA for 'startle intensity': $F(2,90) = 411.2$, $p < .001$; no interactions with 'genotype' or 'treatment', all *p's* > .05; Figure 21A]. Also, all mice regardless of experimental condition displayed decreasing ASR across the three blocks of five 120 dB pulses each, confirming that all mice habituated to the 120 dB startle pulse, regardless of genotype or treatment [RM ANOVA for 'startle block': $F(2,90) = 25.4$, $p < .001$; no interactions with 'genotype' or 'treatment', all *p's* > .05; data not shown].

3.5.2 Prepulse inhibition

Three-way RM ANOVA found that as prepulse intensities increased, the %PPI (averaged across ISI) of all mice became more robust ['prepulse intensity': $F(2,90) = 166.7$, $p < .001$; Figure 21B]; i.e. sensorimotor gating increased with prepulse intensities. Importantly, a significant 'genotype' by 'prepulse intensity' interaction [$F(2,90) = 8.3$, $p < .001$; Figure 21B] was found as well as a genotype difference for the average %PPI [$F(1,45) = 5.5$, $p = .02$; Figure 21C]. Two-way ANOVA was used to investigate this interaction, with data split by 'prepulse intensity'. Significant effects of 'genotype' were found for %PPI at prepulse intensities of 82 dB [$F(1,45) = 9.8$, $p = .003$] and of 86 dB [$F(1,45) = 9.3$, $p = .004$] (with $p > .05$ at 74 dB; Figure 21B) with *APPxPS1* females showing reduced prepulse inhibition compared to WT females. CBD treatment had no overall effect on sensorimotor gating and also did not change the genotype effect (i.e. no overall 'treatment' effect and no 'genotype' by 'treatment' interactions for any prepulse intensity; all p's $> .05$).

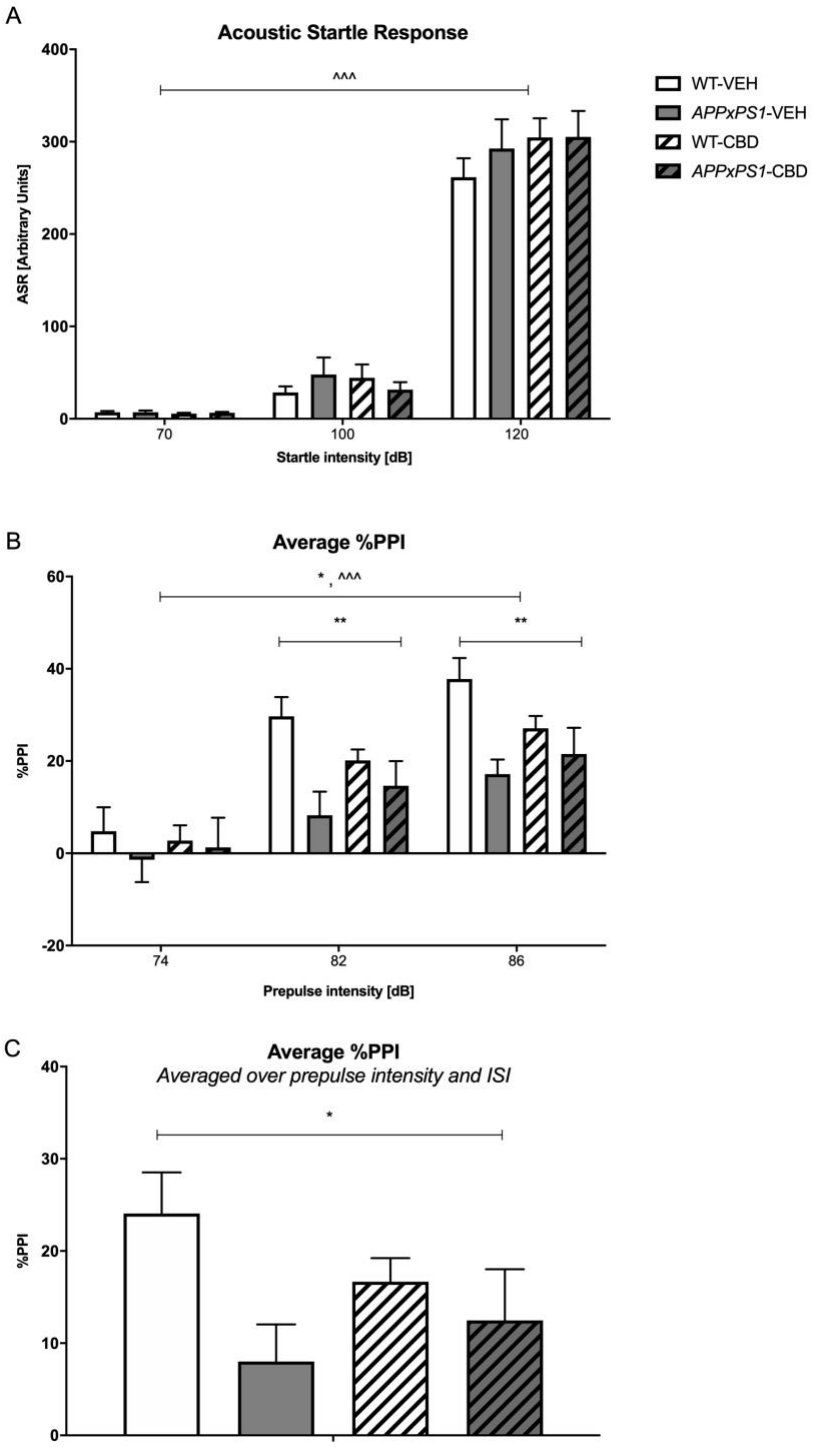

Figure 21A-C: Acoustic startle response (ASR) and sensorimotor gating (PPI). **A)** ASR to increasing startle pulse intensity (70/100/120 dB), **B)** percentage prepulse inhibition [%PPI] averaged over trials for increasing prepulse intensities (74/82/86 dB) and **C)** %PPI averaged over prepulse intensity and interstimulus interval (ISI). Data for non-transgenic wild type-like (WT) control and double transgenic APPSwe/ PS1ΔE9 (APPxPS1) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant 'genotype' effects are indicated with '*' (*$p < .05$) and RM effects are indicated by '^' (^^^$p < .001$).

Chapter 4: Discussion and Conclusions

4.1 Summary

The research conducted as part of this thesis investigated for the first time the behavioural effects of chronic administration of 5 mg/kg CBD in 12-month-old female double transgenic *APPxPS1* mice. Behavioural differences in *APPxPS1* mice compared to WT mice were detected and CBD impacted upon some of those differences. Namely, the main findings were; i) *APPxPS1* mice exhibited a hyper-locomotive phenotype in the LD test regardless of treatment; ii) CBD treatment increased *rearing* of both WT and *APPxPS1* mice in the dark zone of the LD test; iii) *APPxPS1* mice spent more time in the dark zone compared to WT littermates (and tended to travel further in the dark zone); iv) CBD-treated *APPxPS1* females were the only mice to show a preference for the dark zone; v) all mice showed similar motor abilities in the pole test and in the accelerod with the exception that *APPxPS1* mice regardless of treatment fell off the accelerod sooner in their worst trial compared to WT mice; vi) *APPxPS1* mice developed a deficit in object recognition memory in NORT, which was reversed by CBD treatment; vii) *APPxPS1* mice although being slower than WT mice, showed intact spatial learning and memory but exhibited impaired perseverance in the CB probe and rCB probe; viii) the ASR of all mice was similar but *APPxPS1* transgenic females regardless of treatment showed a deficit in PPI. Thus, most important for the study rationale was the finding that CBD treatment reversed the cognitive deficits of *APPxPS1* transgenic females in the object recognition memory test.

Wandering, restlessness and agitation are commonly seen in patients of AD, as previously reviewed (Alzheimer's Association 2018; AIHW 2012). Accordingly, task-dependent hyper-locomotive behaviours have been reported in the *APPxPS1* model

previously in both 7-month-old male and female mice (Cheng et al. 2013; Cheng et al. 2014b), and were replicated in this study in the LD test. *APPxPS1* females displayed increased locomotion in the entire arena of the LD test compared to WT littermates, and CBD treatment did not affect this. Importantly, this hyper-locomotion was also evident when analysing locomotion in the dark zone alone, indicating that anxiety levels did not impact upon the hyper-locomotive phenotype of *APPxPS1* mice, as behaviours in the less aversive dark zone are free of state (spatio-temporal) anxiety (Crawley 1985; Ramos 2008).

CBD had no effect on the locomotion of WT mice nor on the hyper-locomotive phenotype of *APPxPS1* mice. This is in line with other studies which found that, e.g. acute administration of 60 mg/kg CBD and acute dosing with 10 mg/kg CBD had nil effect on the locomotion of WT male Swiss mice (Moreira & Guimarães 2005) and male C57BL/6 mice respectively (Todd & Arnold 2016). Furthermore, 1, 5, 10 and 50 mg/kg CBD given acutely or for 15 days or 21 days also did not affect the locomotion of male C57BL/6JArc mice (Long et al. 2010). Although CBD did not affect the hyper-locomotive phenotype of *APPxPS1* mice in this study, it is interesting to note that CBD can impact on hyper-locomotion evident in other mouse model systems. For example, acute administration of 60 mg/kg CBD has been found to effectively reduce ketamine- (an NMDA-receptor antagonist) and amphetamine- (an indirect dopaminergic agonist) induced hyper-locomotion of male Swiss mice (Moreira & Guimarães 2005), and 1.25-20 mg/kg CBD given acutely also reduced ischaemia-induced hyper-locomotion in gerbils (Braida et al. 2003). The mechanism by which CBD altered these drug-induced and ischaemia-induced hyper-locomotion is not clear (Braida et al. 2003; Moreira & Guimarães 2005), and the mechanism by which the *APPxPS1* transgene induces hyper-locomotion in *APPxPS1* mice has not yet been studied. It is feasible that the

mechanism inducing the hyper-locomotion in *APPxPS1* mice is distinct from the mechanism by which CBD reversed the hyper-locomotion in the mentioned studies, which would explain why CBD did not have an effect on the hyper-locomotive phenotype of *APPxPS1* mice in this study. Thus, more research into the possibility of CBD reducing *APPxPS1* hyper-locomotion is required.

Exploration was also examined in the LD test. CBD treatment increased the frequency of *rearing* of both WT and transgenic mice specifically in the dark zone, indicating that the mice treated with CBD were more inclined to explore in the less aversive dark zone than their vehicle-treated counterparts. A previous study found that *rearing* was unaffected in the open field (OF) test after both acute and chronic administration of 1, 5, 10 and 50 mg/kg body weight CBD in 12-14-week-old male C57BL/6JArc mice (Long et al. 2010). Interestingly, an acute dosage of 50 mg/kg CBD increased the OF exploration of mutant mice in a *neuregulin 1* mouse model of schizophrenia (C57BL/6JArc background, males aged 21 ± 3 weeks), however, this effect disappeared after chronic CBD treatment (Long et al. 2012). This indicates that the current study revealed new insights into the complex, potentially task- and strain-specific effects of CBD on exploration. Furthermore, mice of the previously mentioned studies were much younger (and also male) so the age at time of treatment and sex could potentially be another reason for the differences between the current and past studies.

Anxiety was also measured in the LD test. *APPxPS1* mice showed an aversion to the more anxiety-producing light zone more so than their WT counterparts without CBD impacting on this, which is suggestive of an anxiogenic phenotype of *APPxPS1* females. It was important to determine the anxiety phenotype of these mice as stress and anxiety can impact upon the behavioural performance of experimental mice

(Brinks et al. 2007; Harrison, Hosseini & McDonald 2009), and could have therefore been a confounding factor for the other tests of cognition in this study; therefore anxiety was investigated here as a control measure. In particular, any effect of the *APPxPS1* transgene or of CBD on anxiety in this study needed to be determined to ensure that the subsequent behavioural results found were true indications of the test outcome, and not the result of a confounding anxiety phenotype of *APPxPS1* mice or of the effect of CBD on anxiety. Thus, as this study found that the *APPxPS1* transgene affected anxiety in the experimental mice, behavioural results in latter tests had to be interpreted accordingly. Furthermore, it is known that anxiety can be present in AD patients (Alzheimer's Association 2018) and is reduced in *APPxPS1* mice (Cheng et al. 2014b; discussed further below), adding further to the rationale to anxiety-related behaviours in the current study.

As mentioned, *APPxPS1* mice showed an aversion to the light zone more so than WT mice, suggesting that *APPxPS1* females had an anxiogenic phenotype. This is contradictory to the findings of a previous study, in which 7-month-old female *APPxPS1* mice spent more time in the light zone, indicative of an anxiolytic phenotype (Cheng et al. 2014b). Importantly, the interpretation of the anxiety-related results in the LD paradigm in this study is confounded by the finding that the control WT mice did not show the expected, and previously reported preference for the less aversive dark zone (Cheng et al. 2014b). Instead, WT mice of both treatment groups failed to develop a preference for the dark zone. This complicates findings as it suggests that the paradigm failed to induce state anxiety in the control mice as it should have; hence, any results found for either experimental condition of treatment or genotype for anxiety should be interpreted with caution.

In accordance with the above, while this study found that CBD had no effect on anxiety across both WT and *APPxPS1* mice, these results need to be interpreted carefully. Previous preclinical studies have shown that chronically administered CBD can produce anxiolytic-like effects. For example, administration of 20 mg/kg CBD daily for 6 weeks in male and female C57BL/6J mice resulted reduced EPM anxiety compared to vehicle-treated mice (this was a task-specific effect as no effects on anxiety were found for the LD test or OF; Schleicher et al. (2019)). However, other studies indicate that acute, chronic and long-term administration of CBD has no effect on anxiety. For example, a single dose of 10 mg/kg CBD had no effect on OF anxiety of 14-week-old male C57BL/6 mice (Todd & Arnold 2016), and chronic daily administration of 20 mg/kg CBD had no effect on the levels of EPM anxiety in 24 ± 1 weeks old male WT and *APPxPS1* mice (Cheng et al. 2014a). Furthermore, long-term oral administration of the same dosage of CBD also had no effect on 10-month-old male WT and *APPxPS1* mice (Cheng et al. 2014c). Furthermore, it has been suggested that the anxiolytic effects of CBD may only be evident after an external stressor has been applied, for example, following daily unpredictable stress (Campos et al. 2013). Accordingly, it is evident that the effect of chronic CBD on anxiety appears to be complex and likely to be dose- and paradigm-specific. Interestingly, *APPxPS1* mice chronically treated with CBD were the only mice with a preference for the dark zone, suggesting an anxiogenic effect of CBD specifically for this experimental group. However as mentioned, care must be taken in this interpretation since the LD paradigm did not work for the WT mice. Nonetheless, CBD is known to exhibit a biphasic dose-response in relation to anxiety in both humans and in animals including experimental mice (Rey et al. 2012; Zuardi et al. 2017). For example, an acute dosage of 300 mg CBD prior to an anxiety-inducing task (public speaking) reduced subjective anxiety

measures in healthy human subjects following the test, while 100 mg and 900 mg of CBD did not (Zuardi et al. 2017). Thus, it is possible that while in the Cheng et al. (2014a) a higher dosage of 20 mg/kg CBD did not affect anxiety in the *APPxPS1* model, the low dosage of 5 mg/kg CBD in this study increased anxiety in the transgenic mice due to the biphasic properties of CBD on anxiety (although more research would be required as to why the anxiety of WT mice treated with CBD was unaffected). Furthermore, a different paradigm, and age and gender of mouse were used in these studies, which could contribute to the difference in findings here.

Motor function impairment is not always reported in patients with AD, although it has been more recently considered as an associated non-cognitive symptom of the disease (Buchman & Bennett 2011). In our study, all mice performed equally well in the pole test. In the accelerod, *APPxPS1* mice fell off the accelerod sooner than WT mice on their worst performing trial, however, averaged across the two days, all mice showed similar motor performance regardless of the experimental condition. This moderate deficit is potentially due to an increase in slipping off the rod, rather than due to a distinct deficit in motor coordination and balance as the best performance was similar across experimental groups. Supporting this idea is the finding from a previous study in which male and female *APPxPS1* mice at 6 months of age tended (not significant) to slip more often than WT mice in the balance beam test (Kuwabara et al. 2014). However, a more reliable measure of motor function is looking at the average across a number of trials (Kovács & Pearce 2013) and perhaps of the best performing trial. As stated earlier, these measures of motor performance were not different between WT and *APPxPS1* transgenic mice. This is in line with the majority of previous findings that there is no motor phenotype in this model when using an accelerating rotarod paradigm (Kemppainen et al. 2014; Lalonde, Kim & Fukuchi

2004). For example, 7-month-old male and female *APPxPS1* mice have been found to show intact motor abilities in an accelerating rotarod paradigm, and in the grip strength, stationary beam and coat hanger tests (Lalonde, Kim & Fukuchi 2004), and 3.5-month-old male *APPxPS1* mice had wild type-like function in an accelerod paradigm (while *APPxPS1* mice with induced transient global ischaemia did not; Kemppainen et al. (2014)). However, other studies have found conflicting evidence as to the presence of motor deficits in the *APPxPS1* model. For example, 6-month-old males and females had significant motor deficits in a non-accelerating rotarod paradigm (Kuwabara et al. 2014). It seems that the conflicting evidence in part may be due to task-specific findings; those using a non-accelerating rotarod found a motor deficit in *APPxPS1* mice (Kuwabara et al. 2014), while those using an accelerating paradigm (including this study) did not (Kemppainen et al. 2014; Lalonde, Kim & Fukuchi 2004). Non-accelerating paradigms are more so a test of endurance, while accelerating rotarod paradigms are a more reliable test of motor function (Deacon 2013). Further, the rate of acceleration in accelerating paradigms must be controlled to prevent fatigue confounding the results. Thus, the differences in the two rotarod paradigms could explain the conflicting evidence as to the motor function of *APPxPS1* mice.

Moreover, acute and chronic dosages of CBD have previously demonstrated few extrapyramidal side effects on motor function (Iffland & Grotenhermen 2017). Accordingly, chronic CBD had no impact upon the ability of mice to complete the pole test or accelerod paradigms in this study, indicating that CBD had no adverse side effects on the motor function of WT or *APPxPS1* transgenic mice. This is in line with previous studies. For example, 20, 40 or 80 mg/kg CBD administered 1, 2 or 4 h prior to rotarod testing did not have any effect on motor performance in male Swiss mice

(Ten Ham & De Jong 1975). Also, 50, 100 or 200 mg/kg CBD administered 1 h prior to accelerod testing in adult male Wistar Kyoto rats had no effect on motor function (aside from the finding that 100 mg/kg CBD affected the number of foot slips of the rats; Jones et al. (2012)). Also, in human patients with Parkinson's disease, 4 weeks of daily treatment with a flexible dosage of oral CBD (starting at 150 mg) in addition to usual therapy did not worsen motor function symptoms (Zuardi et al. 2009).

Recognition impairments are common symptoms of AD, particularly in the advanced symptomatic stage, with patients often having issues recognising faces and objects (Alzheimer's Association 2018). Accordingly, the current study detected a novel object recognition deficit in 12-month-old *APPxPS1* females in the NORT paradigm. This finding has been demonstrated in the *APPxPS1* model before with 5-6-month-old males developing novel object recognition impairments (Cheng et al. 2014a). Furthermore, object recognition impairments in female *APPxPS1* mice have been reported to be robustly evident at 12 months of age in the two object recognition test (within a V-maze, rather than a square arena as in the NORT), which correlates with the advanced symptomatic stage of AD (Aso, Andrés-Benito & Ferrer 2016). It is important to note that object recognition memory deficits of *APPxPS1* transgenic mice appear to be sex- and test protocol-dependent. For example, as mentioned above Cheng et al. (2014a) found object recognition impairments in males in the NORT paradigm in a square arena, yet Cheng et al. (2014b) and Jardanhazi-Kurutz et al. (2010) found that 7-month-old and 12-month-old females had intact object recognition memory, respectively, as demonstrated by a preference of greater than 50% for the novel object in the NORT, (although the preference for the novel object in the latter study was reduced compared to the control mice). This highlights a difference in the recognition memory between the two sexes as found in the same paradigm. Also, when

using the two object recognition test in a V-maze, Aso, Andrés-Benito and Ferrer (2016) found a recognition impairment in 12-month-old females as mentioned, showing that the paradigm used affects the observance of recognition deficits. Thus, this study is the first to find a robust memory impairment in the NORT paradigm for 12-month-old female *APPxPS1* mice.

Importantly, chronic treatment with CBD was able to rescue the novel object recognition deficit in the current study, i.e. CBD-treated transgenic mice developed a preference for the novel object. Thus, low dose CBD was able to reverse the object recognition deficit of 12-month-old *APPxPS1* females, in line with earlier studies using a higher dosage of 20 mg/kg body weight CBD in 5-6-month old males (Cheng et al. 2014a). Interestingly, impairments to object recognition have been linked to glutamatergic dysfunction and inhibition of the glutamate transporter 1 (Tian et al. 2019), with preclinical studies showing that antagonism of the glutamate NMDA receptor via memantine can improve object recognition memory (Scholtzova et al. 2008). Importantly, CBD has been found to interact with NMDA receptors (Hallak et al. 2011), indicating that CBD may have reversed the object recognition deficits of *APPxPS1* mice in this study through the glutamatergic system.

Spatial disorientation is another symptom commonly seen in patients with AD (Alzheimer's Association 2018). Accordingly, spatial learning and memory of *APPxPS1* mice was investigated in this study in the CB paradigm. The current study found that during the training phase of the CB, all mice acquired the task (learned the position of the baited well) for both CB and rCB training. *APPxPS1* mice learned in a similar way as WT mice did, which is evidenced by a paralleled decrease in the latency to find the reward over days for both genotypes. This indicates intact spatial learning abilities for *APPxPS1* females. Transgenic mice took generally longer per day to find

the position of the food reward which could be related to a motor deficit or a reduction in the motivation of the mice to find the reward (Zhang et al. 2011). Analysing the CB test in more detail revealed that the average speed of mice on the board during CB training and rCB training was reduced in *APPxPS1* mice. The differences seen in speed appear unlikely to be linked to varying levels of motivation to find the food reward. All mice showed motivation to consume the food reward during habituation, and the few mice, which refused to drink the food reward during training were not of a particular genotype. Importantly, the CB is known to be a less-stressful alternative to the MWM (Grech et al. 2019), which is significant in this study as we found an anxiogenic-like response of the *APPxPS1* mice in the LD test; thus, anxiety should not have impacted upon the learning of mice either. Also, chronic CBD had no effect on spatial learning, and importantly has previously been shown to have no effect on memory in control animals (Fagherazzi et al. 2012).

We also investigated intermediate-term and long-term memory of the mice in CB and rCB training, as defined by the ITI employed and outlined already in section 2.3.5, i.e. the intermediate-term memory of mice was determined by the average latency of trial 2 and 3 (~ 20 min following the previous trial), and the long-term reference memory of mice was determined by the latency of trial 1 only (~24 h following the last trial of the day prior). Short-term, intermediate-term and long-term memory each have temporal constraints and have distinct molecular characteristics. For example, short-term memory transpires after only a few minutes and is independent of protein synthesis and gene transcription, intermediate-term memory occurs over several hours and is dependent on protein synthesis but not gene transcription, and long-term memory occurs after a period of 24 h and is gene transcription-dependent (Kandel 2001; Stough, Shobe & Carew 2006; Taglialatela et al. 2009). Thus, the interval used

between training trials (i.e. 20 min between trials of each day, and 24 h between the last and first trial of successive days) can inform which kind of memory impairment is present and therefore which molecular mechanism is impaired, if any (Taglialatela et al. 2009). Thus, when considering the intermediate-term memory and the long-term reference memory of mice in this study, it became apparent that, similarly to the overall learning performance, all mice showed a decrease in the latency to find the reward across days, and *APPxPS1* mice took longer per day to find the food reward. Thus, learning performance based on intermediate-term memory and reference memory was not affected by the *APPxPS1* genotype during both CB and rCB training. Also, CBD had no effect on the two types of spatial learning of mice in both the CB and the rCB training sessions. Investigating CB learning in terms of intermediate-term and long-term memory is relatively new and has only recently been described in our laboratory in a mouse model of motor neuron disease (Kreilaus et al. 2019); thus, this is the first study to identify that the intermediate-term and long-term retention learning memory of 12-month-old *APPxPS1* females is wild-type like.

In the CB and rCB probe, all mice recognised their target zone above chance for the 2 min duration of the trial (there was only a weak trend for a target zone preference in *APPxPS1*-CBD mice in the rCB probe; this was most likely driven by a large SEM due to a statistical outlier, rather than a deficit in memory performance. The statistical outlier was not excluded from analysis as it performed the task properly when the test video was reviewed). This indicates that *APPxPS1* mice did not have a spatial memory impairment, which is converse to what has been found in the past in the CB and other tests of spatial memory. For example, the CB has previously been shown to reliably detect cognitive deficits in 8-9-month-old *APPxPS1* females, which did not show a preference for the target zone during the rCB probe (Cheng et al. 2014b). *APPxPS1*

mice also show spatial learning and retention deficits in other tests of spatial memory. For example, 12-month-old *APPxPS1* female mice (on a C57BL/6 background) show a reduced latency during training to find the platform in the hidden MWM, and also spend less time in the target zone during the probe trial (Zhang et al. 2011). 18-month-old male *APPxPS1* mice have demonstrated spatial impairments in the early stages of training in the MWM, with less accurate memory for the platform location on the first day of training, and also towards the end of training as they travel nearly twice as far as WT mice to find the platform (Savonenko et al. 2005). 16-month-old *APPxPS1* mice of both sexes have also demonstrated impairments in the Barnes maze, whereby they showed impaired acquisition learning and were less likely to use a spatial search strategy compared to WT mice, and also showed a deficit in memory in probe tests (O'Leary & Brown 2009). However, it is important to note that caution should be used when comparing the findings from studies using the MWM and Barnes maze with findings from studies using the CB, as the motivation involved in the task is different, i.e. the motivation to avoid drowning and to escape are utilised in the MWM (Grech et al. 2019) and the Barnes maze (O'Leary & Brown 2009), while positive reinforcement is used in the CB. Thus, it is clear that spatial memory impairments have been found in this model before, and it is especially important to suggest why this study did not find the same spatial memory impairments that Cheng et al. (2014b) did in *APPxPS1* females in the CB (since the same paradigm was used). Specific protocols can impact upon results of the same test (Post et al. 2011), and it is important to note that there were significant differences between this study and Cheng et al. (2014b). Firstly, the laboratories in which these experiments were conducted were different, and secondly the experimenter in this study was female, while the experimenter in Cheng et al. (2014b) was male. Differing laboratory and experimenter conditions can

have an impact on the baseline behaviour of mice (Lewejohann et al. 2006), with evidence showing that male-related stimuli can cause physiological stress in rodents (Sorge et al. 2014). Evidence of these differences between the current study and Cheng et al. is the fact that the 12-month-old WT mice in this study only required 5 days of CB training and 4 days of rCB training to acquire the task, while those in Cheng et al. received 9 days of CB training and 4 days rCB training, i.e. mice in the Cheng et al. study required longer training. It is speculated that this may be caused by the difference between the laboratory and experimenter of each study, with the learning of the task made harder for the mice in the Cheng et al. study due to the male-related stimuli and differences in cue presentation and noise in the facility, thereby altering the protocol of the CB, which could account for the differences found for spatial memory.

To further investigate the spatial memory in these mice, we took a relatively novel approach for CB analysis and examined the preference of mice for their target zone in the first 30 s and the second 30 s to identify retrieval memory and perseverative behaviour, similar to what was outlined in a recent study (Grech et al. 2019). Briefly, and as outlined in section 2.3.5, mice that showed a preference for the target zone in the first 30 s are suggested to have intact retrieval memory, while those that do not have a deficit. A preference for the target zone over next 30 s indicates persistence to find the food reward, while no preference indicates either i) lack of persistence or ii) cognitive flexibility in adaptation to the lack of food reward (since the probe trial can be considered as an extinction trial; Grech et al. (2019)). The current study found that all mice regardless of experimental condition exhibited intact retrieval memory in the CB and rCB probe trial. CBD had no effect on retrieval memory. When analysing the memory performance in the second 30 s of the CB and rCB probe trials, only WT mice showed a preference for their target zone, indicating perseverance to find the food

reward. However, *APPxPS1* mice of both treatment groups no longer showed a preference for the target zone, which as proposed could be an indication of cognitive flexibility in adaptation to the lack of the food reward (Grech et al. 2019), or simply a lack of persistence. As this is a very novel approach at investigating the spatial memory of mice in the CB, further investigation is required to determine exactly what the lack of a preference for the target zone in the second 30 s means. Future analysis of search patterns, i.e. allocentric (using external cues or landmarks in relation to each other to navigate, independent of self) versus egocentric (based on directional (left-right) responses to navigate, independent of environmental cues) search strategies, could help answer this.

Sensorimotor gating is the filtering of extraneous information and can be assessed in both humans and in animal models in PPI paradigms, and was assessed in this study as there is some evidence that PPI is affected in AD. The present study found that the average %PPI of *APPxPS1* females was reduced compared to littermates, indicating a robust PPI deficit. This is in line with Wang et al. (2012) which found robust PPI deficits as early as 7 months in female *APPxPS1* mice (generated on mixed B6SJLF1 x B6D2F1 x Swiss Webster background), which were associated with cerebral amyloid neuropathology. However, these findings are converse to the study by Cheng et al. (2014b) which found that PPI was largely unaffected in female 46 ± 1 week old female *APPxPS1* mice, with deficits found at the 128 ms ISI only. Furthermore, analysing the PPI response in more detail, the genotype effect was evident at the more robust prepulse intensities of 82 dB and 86 dB, which are significantly above the background noise. Similar to Wang et al. (2012), the PPI deficit of *APPxPS1* females was not accompanied by any changes in the baseline startle response or habituation thereof. The conflicting preclinical evidence that PPI is affected in mouse models of

AD is also seen in clinical trials in AD patients. This conflict in evidence may be due to studies differentially grouping patients with MCI together with patients with AD, versus analysing these groups separately. For example, patients with mild AD or MCI (grouped together) were shown to have normal PPI in a passive acoustic PPI paradigm compared to healthy controls (Hejl et al. 2004), but in another study testing acoustic PPI, patients with 'early dementia of Alzheimer's type' had a gating deficit, while those with MCI (analysed as a separate group) had gating facilitation. Furthermore, a study using a double-click paradigm found that AD patients have suppression of the P50 event-related potential following the second click (a sensory gating deficit; Jessen et al. (2001)). It has been suggested that PPI deficits in early stage AD patients could be due to disturbed regulation of the limbic cortico-striato-pallido-pontine circuitry (Ueki et al. 2006), or due to cholinergic dysfunction and alpha-7 nicotinic receptor loss (Jessen et al. 2001),

Chronic 5 mg/kg CBD did not affect the ASR or PPI of experimental mice and also did not reverse the PPI deficit in *APPxPS1* transgenic mice. Previous studies have demonstrated that acute CBD is able to attenuate pharmacologically induced disruptions of PPI, without affecting any aspects of sensorimotor gating in control mice. For example, pretreatment (single i.p. injection) of 30 and 60 mg/kg CBD reduced the PPI deficits in amphetamine-treated mice (Pedrazzi et al. 2015), and a single dose of 5 mg/kg CBD reversed PPI deficits induced by the noncompetitive NMDA receptor antagonist MK-801 (Long, Malone & Taylor 2006). These studies suggest that CBD may have some capabilities to reverse particular PPI deficits without side effects. However, other studies have found no beneficial effects of CBD on PPI deficits. For example, 3 and 10 mg/kg CBD administered to an MK-801-induced rat model of schizophrenia was unable to recover PPI deficits and actually caused PPI

deficits in the control group (Gururajan, Taylor & Malone 2011). Another study administering 20 mg/kg CBD i.p. daily for 8.5 weeks caused a PPI deficit at prepulse intensities of 75 dB and 80 dB in 6-month-old C57BL/6J mice, suggesting a possible side effect of the drug (Schleicher et al. 2019). The discrepancies in the findings of CBD effects on PPI may be due to the fact that PPI test outcomes are heavily dependent on the protocol characteristics used in each study (Karl et al. 2011), with importance being placed on the ISI, duration and intensity of the prepulse and startle pulse, the total protocol duration and the particular algorithms for calculating PPI and interpretation of results, as well as habituation procedures prior to PPI testing (Swerdlow, Braff & Geyer 2000). Furthermore, the effect of CBD on PPI in the mentioned studies were investigating the effects of acute CBD, rather than chronic administration of CBD as investigated in this study. Thus, different protocol characteristics used in each study may account for the differing results found, i.e. why CBD did not reverse *APPxPS1* PPI deficits in this study when it has shown the potential to previously.

4.2 Limitations and future perspectives

Some limitations affected this study. Firstly, the present study did not investigate male mice for feasibility reasons. It is known that there is a sexual dimorphism profile of the AD-relevant pathologies (Aβ, NFT and pro-inflammatory cytokines) for this model (Jiao et al. 2016) and behavioural differences between male and female *APPxPS1* mice (Cheng et al. 2013; Cheng et al. 2014b). Secondly, this study is limited in that it only investigated the effects of CBD in an animal model of amyloidosis without an investigation of the effects of CBD on tau pathology. Future investigation could include a tau pathology animal model, or a model with both amyloid and tau

pathology, for a more comprehensive investigation of the therapeutic potential of low dose CBD for AD. Also, future studies could investigate the effects of chronic CBD administration in *APPxPS1* mice at an earlier age (relevant for early stage or pre-AD), to determine the effectiveness of CBD treatment prior to AD symptom onset, or could investigate higher dosages of CBD.

There are numerous other aspects of this study that can be followed up in future studies. Firstly, further analysis into the search patterns of mice during the CB and rCB could be conducted to determine any deficits in allocentric or egocentric navigational strategies that might explain the lack of perseverance in *APPxPS1* mice during probe trials. Secondly, while the brain tissue of test mice was collected at the conclusion of behavioural testing, it was not analysed as part of this thesis due to time constraints. An investigation of the neuropathological markers of AD in the test mice to determine the effect of 5 mg/kg body weight CBD at the molecular level in the *APPxPS1* mouse model of AD will likely be performed as a follow up study to this thesis. It is likely that quantification of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the PFC, hippocampus and cerebellum will be determined through ELISA techniques in this future investigation. Future analysis would also investigate levels of IBA-1 to determine microglial activity, and levels of TNF-α and IL-1β as markers of neuroinflammation. These molecular investigations would add insight into the effect of chronic 5 mg/kg CBD on the molecular markers of AD in the *APPxPS1* model.

A major limitation of this study was the failure of the LD test to induce state anxiety in the WT mice. Numerous aspects of this study's design of the LD test were reconsidered to determine an explanation for this failure of WT mice to show a preference for the dark zone. Firstly, it was hypothesised that perhaps the mice were showing risk-assessment behaviour around the passage opening between the two

zones, distorting the ratio of the time spent in either zone. To assess the time spent and distance travelled in the more distal parts of the zones, a central exclusion zone was created in the analysis software, resulting in three zones. The central exclusion zone data was excluded from analysis, with the new, smaller light and dark zones used when reanalysing anxiety behaviours in the LD test. However, this reanalysis did not alter the previous findings, suggesting that the activity of mice in the central exclusion zone did not cause the unexpected findings; thus, the failure for the WT mice in this test is due to some other factor.

In this context it is important to mention that in line with other experiments in our laboratory, tests of anxiety are generally conducted at the start of a test battery, so that test mice are naïve with only a limited amount of handling prior to anxiety-related testing in the LD paradigm. This is important because the handling of experimental animals during testing is a source of stress that can impair their responses (Gouveia & Hurst 2017). Furthermore, it is known that the changing of cages of test mice can increase general activity and stress levels (Bailey & Crawley 2009), therefore, animal husbandry was not carried out on experimental days or only after testing had been completed for that day. Furthermore, as anxiety was one of the main parameters being assessed in the LD paradigm, mice were given a full hour of habituation to the testing room prior to the LD to reduce any stress caused by transport from the animal housing facility (Bailey & Crawley 2009). Thus, while all aspects of the LD test were carefully considered, an explanation for the failure of WT mice to show a preference for the dark zone is unable to be given.

4.3 Conclusion

This study found that 12-month-old female *APPxPS1* transgenic mice were hyper-locomotive and showed no motor deficits. Spatial learning and memory were not affected in AD transgenic females, but transgenic mice showed object recognition deficits and PPI deficits. Chronic administration of 5 mg/kg CBD instigated exploration in the dark zone of the light dark test but did not affect motor function or spatial learning and memory and did not reverse PPI deficits evident in transgenic females. Importantly, 5 mg/kg CBD reversed novel object recognition deficits in *APPxPS1* transgenic females suggesting a therapeutic-like effect in this established mouse model for AD. Future research into the molecular effects of CBD should be considered, as well as investigating other doses and age of test animal. In conclusion, this study suggests that CBD has therapeutic value for particular behavioural impairments present in AD patients.

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